Effects of FTY720 in MRL-lpr/lpr Mice: Therapeutic Potential in Systemic Lupus Erythematosus

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ABSTRACT ***Objective.*** To examine the effects of a novel immunosuppressant, FTY720, on hematolymphoid cells and the clinical course of MRL-lpr/lpr (MRL/lpr) mice genetically predisposed to systemic lupus erythematosus (SLE).

Methods. Apoptosis of hematolymphoid cells was determined *in vitro* by FACScan after staining with propidium iodide or merocyanine 540. From 4 months of age, 15 female MRL/lpr mice received oral administration of 2 mg/kg each of FTY720, methylprednisolone (mPSL), or vehicle, 3 times per week. Therapeutic efficacy was evaluated by levels of anti-dsDNA antibodies in serum and the survival rate. In parallel, T cell proliferation and secretion of interleukin 2 (IL-2) induced by anti-CD3, phenotypes of the spleen, lymph node and bone marrow cells, as well as immunohistochemistry of the kidney, were examined *in vitro*.

Results. FTY720 at 2 µM induced apoptosis in more than 70% of double negative (CD4–/CD8–) T cells from the spleen of MRL/lpr mice *in vitro*. Oral FTY720 was tolerated well with no apparent side effects. FTY720 treated and control mice gained weight at an identical pace through to 9 months of age. FTY720 significantly suppressed the production of anti-dsDNA antibodies (FTY720 vs control: 1739 ± 898 U/ml vs 410 ± 356 U/ml at 8 months of age; $p < 0.05$) and reduced the deposition of IgG in glomeruli compared to control animals. At 9 months of age, the survival rate in the FTY720 treated mice was 86.9% compared to 33.0% in controls ($p < 0.01$). FTY720 decreased the number of double negative T cells from the spleen and lymph nodes *in vivo*, and increased T cell proliferation and IL-2 secretion induced by anti-CD3 stimulation *in vitro*.

Conclusion. FTY720 suppressed the development of autoimmunity and prolonged the lifespan of female MRL/lpr mice. Suppression of autoimmunity, at least in part, may have resulted from an apoptogenic potential of FTY720. Hence, it could be useful for primary or adjunctive therapy of human SLE. (J Rheumatol 2002;29:707–16)

Key Indexing Terms:
FTY720 LUPUS THERAPY APOPTOSIS

Although the pathogenesis of most autoimmune diseases is yet to be determined, increasing lines of evidence have indicated a role of apoptosis in the development of autoimmune diseases[1]. In several animal models of spontaneous autoimmune diseases, genetic defects causing an abnormal resistance of peripheral T cells to apoptosis have been identified. This applies both to models of systemic lupus erythematosus (SLE) (strains homozygous for the lpr or gld mutations) and to an animal model of type I diabetes mellitus [nonobese diabetic (NOD) mouse]. Thus, the lpr mutation renders the Fas gene inoperative[2]; the gld mutation affects the Fas ligand[3]; and one of the diabetes susceptible loci of the NOD strain maps to the bcl-2 gene[4]. The induction of apoptosis in these mouse models of autoimmunity, therefore, would ameliorate autoimmune manifestations in them. This view is supported by corticosteroid and cyclophosphamide, widely used for the treatment of SLE, which exert immunosuppressive activities, in part, by means of inducing apoptosis in lymphocytes[5]. However, their utility is restricted by their undesirable side effects including infection, premature cardiovasucular mortality, infertility, and neoplasia[6,7]. Immunosuppressive drugs with a high desired/adverse reaction ratio and with a higher specificity for effector lymphocytes causing autoimmunity are awaited.

A potential candidate satisfying such criteria might be FTY720 (2-amino-2-(2-[4-octyl-phenyl]ethyl)-1,3-propanediol hydrochloride), a novel immunosuppressant representing a synthetic analog of a natural product of the ascomycete *Isaria sinclarii*[8]. FTY720 has a chemical structure resembling sphingosine. In this regard, it differs from

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conventional immunosuppressants such as FK506, cyclosporin A, and rapamycin[9]. FTY720 administered *per os* prolongs the survival of skin[10], cardiac[11], and liver[12] allografts in rats, as well as that of renal allografts in dogs[13]. It also prevents the development of graft-versus-host disease in rats, with little apparent toxicity[14]. Although the precise molecular mechanism of the immunosuppressive activity of FTY720 has not been fully elucidated, studies have suggested that FTY720 may induce apoptosis in lymphocytes through activation of phospholipase C[15] or some protein kinases[16], and accelerate homing of lymphocytes to lymphoid tissue by altering the expression of CD62L[17].

Mice of the MRL-lpr/lpr (MRL/lpr) strain spontaneously develop an autoimmune disease resembling human SLE. The disease is characterized by immune complex mediated glomerulonephritis, enlargement of the spleen and lymph nodes, and production of various autoantibodies such as anti-dsDNA antibodies and rheumatoid factors[18]. The MRL/lpr strain exhibits diminished Fas mediated lymphocyte apoptosis, resulting in accumulation of CD3+CD4–CD8– (double negative) T cells[2,19]. These abnormally proliferating T cells do not respond normally to anti-CD3 antibody or mitogens such as concanavalin A, resulting in reduced interleukin 2 (IL-2) production[20].

Suzuki, *et al* reported that FTY720 induces apoptosis in lymphoid organs and prolongs the life of MRL/lpr mice when treatment is started at 4 months of age[21]. We attempted to confirm and extend their findings by evaluating the suppressive effects of FTY720 on the progression of autoimmunity and glomerulonephritis in MRL/lpr mice. Further, the apoptotic potential of FTY720 on lymphocytes was investigated, which may account, in part, for its beneficial effects on autoimmune diseases.

MATERIALS AND METHODS

Mice. Female BALB/c, MRL/MpJ/+/+ (MRL/+), and MRL/MpJ/lpr/lpr (MRL/lpr) mice were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

Collection of blood. Blood was sampled at the start of the experiment and monthly thereafter. It was drawn by puncture of the retro-orbital sinus, allowed to clot for 1 h at room temperature, and kept at 4˚C overnight. Sera samples were then separated by centrifugation and stored at –20˚C until use. Wild-type MRL/+ mice were used as controls in experiments of lymphocyte proliferation and IL-2 production.

Cell preparations. Thymuses, spleens, or lymph nodes were removed, teased apart, and pressed through a mesh using RPMI 1640 containing 2% fetal bovine serum (FBS) to prepare single cell suspensions. Splenic T cells were obtained by passage through a nylon wool column. Bone marrow cells were flushed from femurs and tibias and resuspended in RPMI 1640 containing 2% FBS. Viable cells were counted by the erythrosine B exclusion test[22] and confirmed for viability > 95%.

Reagents and antibodies. FTY720 was synthesized by Taito Co. (Tokyo, Japan) in cooperation with Yoshitomi Pharmaceutical Industries (Osaka, Japan). For experiments *in vitro*, FTY720 was dissolved in RPMI 1640 medium and adjusted to appropriate concentrations in each experiment. For use *in vivo*, it was dissolved in physiological saline. Dexamethasone (DEX) was purchased from Sigma (St. Louis, MO, USA), and unconjugated or fluorescein isothiocyanate (FITC) conjugated anti-CD3 (145-2C11), anti-IgM and phycoerythrin (PE) conjugated Gr-1 (RB6-8C5) monoclonal antibodies (Mab) were from PharMingen (San Diego, CA, USA). FITC or PE conjugated anti-CD4 (L3T4) and anti-CD8 (Lyt-2) Mab were purchased from Caltag Laboratories (Burlingame, CA, USA). Recombinant human IL-2 (TGP-3, 2.4×10^4 units/mg of protein) was a gift from Takeda Pharmaceutical Co. (Osaka, Japan).

Cell culture. Cells were cultured in RPMI 1640 containing 10% (v/v) heat inactivated FBS and supplemented with antibiotics as well as 2-mercaptoethanol (50 µM: RPMI complete) in a humidified chamber with an atmosphere of 95% air and 5% CO_2.

Flow cytometry. Methods for immunofluorescent staining were as described[23]. Single cell suspensions of the spleen, lymph node, and bone marrow cells were stained with fluorochrome conjugated Mab and analyzed in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using Lysis II software (Becton Dickinson). Combinations of FITC-anti-CD3 and PE-anti-CD4 + CD8 Mab were used for dual surface staining to detect CD3+ double negative T cells. Anti-IgM Mab was used to identify mature B cells in the spleen and bone marrow from MRL/lpr mice. To avoid nonspecific staining, the cells were preincubated with a mixture of unconjugated rat anti-mouse CD32 and anti-CD16 antibodies (PharMingen).

Assessment of apoptosis. Experiments were performed in triplicate in 24 well flat bottom plates at a cell concentration of 2×10^5 cells/ml. Apoptosis was measured in hematolymphoid cells cultured *in vitro* for 0, 2, 4, 8, 12, and 24 h. The number of viable cells was determined by staining with erythrosin B at each time point. Apoptosis was confirmed by flow cytometric analysis of cell nuclei stained with propidium iodide (PI; Sigma)[24] and by oligonucleosomal fragmentation of genomic DNA visualized on agarose gels. Briefly, after stimulation, the cells were washed twice in phosphate buffered saline (PBS) containing 0.1% (w/v) NaN_3, and 1 ml of cold 70% ethanol (v/v) was added to the cell pellet. The cells were then vortexed and incubated at 4˚C for 1 h. They were washed twice and resuspended in 0.1 ml of PBS containing 0.1% NaN_3. For DNA staining, 0.1 ml of 1 mg/ml RNase A (Sigma) was added to the cells while mixing, followed by the addition of 0.2 ml of 100 mg/ml PI (Sigma). The cells were incubated at room temperature for 20 min in the dark and then analyzed by FACScan. Apoptotic cells appeared in the < 2 N DNA peak. They were distinguished from necrotic cells by analyzing the light scatter profile. Necrotic cells showed a large decline in forward-angle light scatter and side scatter, while apoptotic cells showed a smaller decline in them. Necrotic cell fragments and cell debris were excluded by an appropriate gate, and data were recorded on a linear scale. At least 10,000 cells were evaluated per sample in most experiments. Apoptotic cells in certain T cell subsets were determined by increased binding with a lipophilic fluorescent dye [merocyanine 540 (MC540); Sigma], as well as by decreased forward scatter by flow cytometry. Staining with MC540 was performed as described[25] with a minor modification. Splenic T cells from MRL/lpr mice were cultured for 12 h. After the cells were surface stained with FITC conjugated anti-CD4 + CD8 Mab, they were resuspended in 100 ml of PBS supplemented with 2% FCS. Then MC540 (5 mg/ml) was added to the suspension, and the cells were incubated at room temperature for 10 min. Stained cells were analyzed immediately by FACScan.

Detection of DNA fragmentation in agarose gels. Splenic T cells (1×10^6) from MRL/lpr mice cultured with 2 µM dexamethasone for 4 h were used as a positive control for DNA fragmentation. Splenic T cells (1×10^6) from MRL/+ or MRL/lpr mice were stimulated as indicated for 4 h and harvested. They were centrifuged and washed once with cold PBS. The cell pellet was resuspended in 0.6 ml of lysis buffer containing 10 mM Tris-HCl, 20 mM EDTA, and 0.25% Triton X-100 (pH 8.0) on ice. After 5 min, the lysate was centrifuged at 15,000 × g for 10 min at 4˚C in an Eppendorf microfuge tube. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was then placed in a separate microfuge tube with EDTA (12.5 mM) and DNase-free RNase (30 mg/ml) at 37˚C. After 15 min,

70 ml of 0.1% (w/v) sodium dodecyl sulfate was added and the sample was incubated at 55˚C for 10 min. DNA was then extracted with an equal volume of phenol:chloroform (1:1) and precipitated at –20˚C in 0.3 M sodium acetate and 50% (v/v) isopropanol for at least 18 h. The pellet was rinsed with 70% ethanol, air dried, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA). The samples were electrophoresed on a 1.7% agarose gel with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). DNA was then visualized by ethidium bromide staining. A 1 kb DNA ladder (BRL) obtained after digestion using *Hinf I* was employed as standard size fragments.

Experimental design for in vivo treatment. Mice were randomly divided into 3 groups, each of which was treated with FTY720, methylprednisolone (mPSL), or none (controls). Fifteen MRL/lpr mice at 4 months of age were given *per os* either 2.0 mg/kg of FTY720 or mPSL 3 times per week, using a curved blunt-end needle inserted into the throat. Control mice were given 0.5 ml of saline. This dose of 2.0 mg/kg FTY720 was adopted from the published report[21]. All experiments were conducted in accord with the guidelines of the Animal Ethics Committee of Jichi Medical School.

Detection of anti-dsDNA antibodies. An anti-dsDNA ELISA kit (MBL, Nagoya, Japan) was used to quantitate IgG antibodies to double stranded DNA in sera from individual mice. The assay was performed according to the manufacturer's instructions and the quantity of anti-dsDNA antibodies was estimated by comparison against the standard curve.

Proliferation of splenic T cells. The method described by Warner, *et al*[26] was followed for estimating the proliferation of spleen cells *in vitro*. Splenic T cells obtained through a nylon wool column were cultured at 5×10^5 cells/well in flat bottom microtiter plates that had been precoated with 5 mg/ml purified anti-CD3 antibodies. Three days later the cells were pulsed with ^{3}H-thymidine (1 mCi/well) (NEN, Boston, MA, USA) overnight, gleaned with a semiautomatic harvester, and counted by scintillation. Results were expressed in the stimulation index (cpm with stimulant) – (cpm without stimulant)/cpm without stimulant. Spleen cells from 4 animals per group were cultured and analyzed separately.

IL-2 production from spleen cells. Spleen cells from the same animals used for proliferation studies were stimulated by immobilized anti-CD3 for 24 h as described above and culture supernatants were collected. Secretion of IL-2 was quantitated by bioassay using IL-2 dependent CTLL-2 cells as described[27]. Recombinant human IL-2 (TGP-3) was used as a standard for each assay, and data were recorded in units/ml of IL-2.

Histology. At the completion of the *in vivo* studies all animals were sacrified for immunohistological analyses. Kidneys were removed, snap frozen in liquid nitrogen, and stored at –70˚C. For immunofluorescence analysis, unfixed sections were stained with FITC conjugated sheep anti-mouse IgG (Cappel, Malvern, PA, USA).

Statistical analyses. The Mann-Whitney U test and chi-square analysis were used for statistical analyses. Differences among group means were considered significant at a value of $p < 0.05$.

RESULTS

Effects of FTY720 on apoptosis in normal BALB/c mice in vitro. Apoptosis was measured in the thymocytes, spleen cells, and bone marrow cells from normal 6-week-old BALB/c mice that had been cultured for 0, 2, 4, 8, 12, and 24 h. Little apoptosis, if any, was seen at time 0 in any of the cell populations, as determined by PI staining (Figure 1). Since the lowest concentration of FTY720 to induce maximal apoptosis of thymocytes cultured for 8–24 h was 2 µM (data not shown), subsequent experiments were performed at this concentration. FTY720 induced a significant increase in apoptosis of the thymocytes (Figure 1A), but not of the spleen cells (Figure 1B). In agreement with published data[28], DEX increased apoptosis in both the thymocytes and spleen cells to a much higher extent than FTY720 at the same concentrations. In sharp contrast, FTY720 induced apoptosis of the bone marrow cells to a greater degree than DEX (Figure 1C). Thus, the apoptoses of hematolymphoid cells with FTY720 and DEX could be induced by a different mechanism.

Effects of FTY720 on in vitro apoptosis of spleen cells from MRL mice. In MRL/+ mice of the same age, the results were essentially similar to those seen in BALB/c mice (Figure 2A), while in age matched MRL/lpr mice, FTY720 induced apoptosis in about 70% of the spleen cells and to the same extent as DEX (Figure 2B). These results suggested that FTY720 may increase the apoptotic cell death in the CD3+CD4–CD8– double negative T cell population predominant in MRL/lpr mice by 6 months after birth. To confirm this, splenic T cells from 6-month-old MRL/lpr mice were stained with MC540 and a mixture of FITC conjugated anti-CD4 and anti-CD8 Mab. DEX increased apoptosis in both the double negative T cells and CD4+ or CD8+ single positive T cells after 12 h in culture, while FTY720 selectively induced apoptosis in the double negative T cells (Figure 3).

Induction of DNA fragmentation by FTY720 in splenic T cells from MRL/lpr mice. To evaluate whether FTY720 induced DNA fragmentation characteristic of apoptosis in short term cultures, the genomic DNA from splenic T cells was separated by gel electrophoresis. For this purpose, splenic T cells were incubated for 4 h, then harvested and the genomic DNA was obtained. A DNA fragmentation pattern serving as control was of splenic T cells from MRL/lpr mice undergoing apoptosis after incubation *in vitro* with 2 µM dexamethasone (Figure 4, Lane 1). Lanes 2 and 3 show the DNA fragmentation pattern in splenic T cells from MRL/+ mice incubated in medium and 2 µM FTY720, respectively, with no DNA fragmentation. The splenic T cells from MRL/lpr mice cultured in medium had little or no DNA fragmentation (Lane 4). In contrast, DNA in the splenic T cells from MRL/lpr mice incubated with 2 µM FTY720 showed a fragmentation pattern characteristic of apoptosis (Lane 5). A *Hinf I* digest of DNA is shown for comparison (Lane 6).

Effects of FTY720 on the clinical course of lupus in mice. *Survival*. Sixteen-week-old female MRL/lpr mice were divided randomly into 3 groups of 15 animals. Each group received orally either 2 mg/kg of FTY720, 2 mg/kg of mPSL, or vehicle alone, 3 times a week, and was observed daily. The FTY720 treated mice gained weight at a pace identical to that of control mice through to 36 weeks of age (Table 1). As shown in Figure 5, FTY720 prolonged the survival of mice at 8, 9, and 10 months of age significantly longer than controls (at 9 mo, 86.7% of FTY720 treatment vs 33.0% of control; $p < 0.01$), to an extent comparable to that with mPSL.

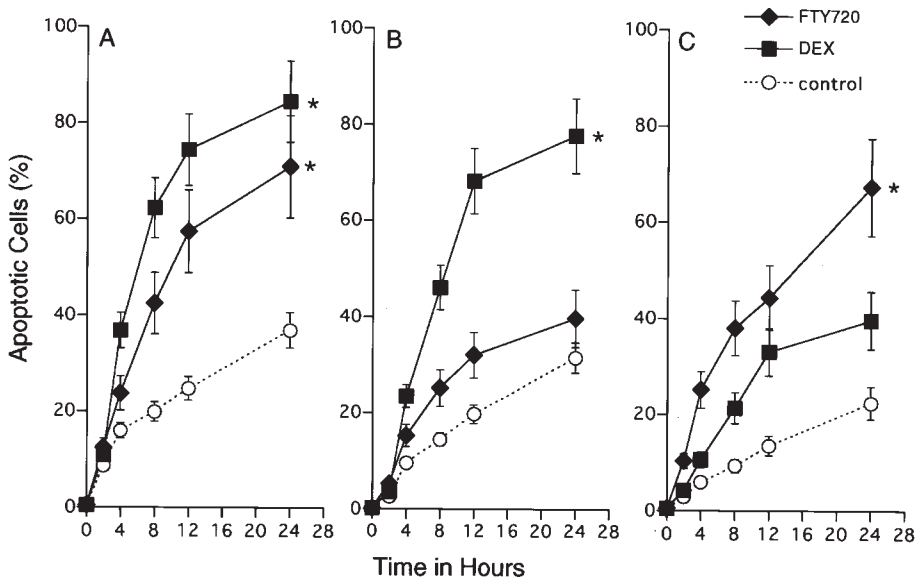

Figure 1. Influence of FTY720 on apoptosis of hematolymphoid cells in normal BALB/c mice. Apoptosis was measured in thymocytes (A), spleen cells (B), and bone marrow cells (C) cultured for various durations in the absence or presence of 2 µM FTY720 or 2 µM DEX. Percentage of apoptotic cells represented by the fraction of hypodiploid DNA was determined by flow cytometric analysis of nuclei stained with PI. Values are mean ± SD. *Significant differences between FTY720 or DEX and controls, $p < 0.05$, Mann-Whitney U test.

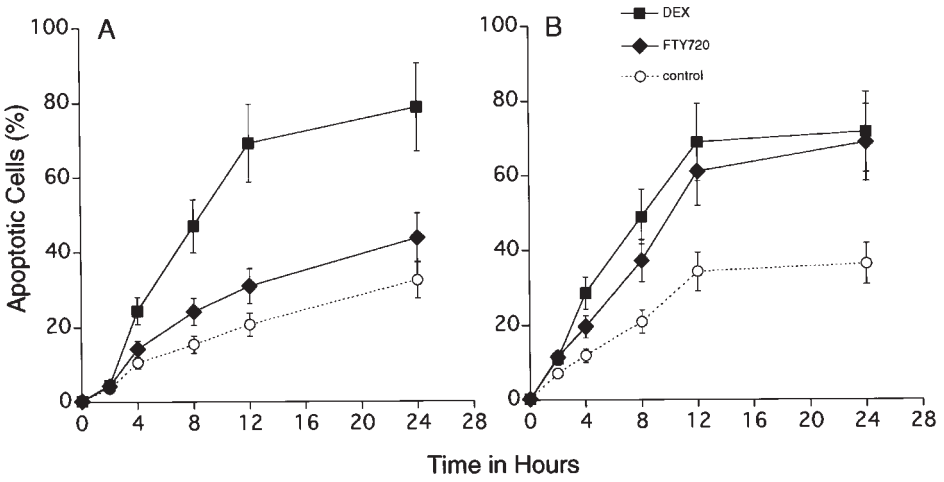

Figure 2. Influence of FTY720 on apoptosis *in vitro* in spleen cells from MRL mice. Spleen cells from 6-month-old MRL/+ (A) or MRL/lpr mice (B) were cultured *in vitro* in the absence or presence of 2 µM FTY720 or 2 µM DEX. Apoptosis was measured by flow cytometric analysis of nuclei stained with PI.

Kidney disease. Frozen kidney sections from 10-month-old mice treated with FTY720 or mPSL were stained with FITC conjugated anti-mouse IgG. As shown in Figure 6, untreated control mice displayed heavy deposits of IgG in the glomeruli, while the majority of FTY720 treated mice showed a marked decrease in IgG deposition. Essentially similar results were obtained when staining for C1q and C3 in the glomeruli (data not shown).

Production of anti-DNA antibodies. Serum IgG anti-dsDNA antibodies were measured in FTY720 treated, mPSL treated, and control MRL/lpr mice at 6 and 8 months of age. There were no significant differences in the levels of serum anti-dsDNA antibodies among the 3 groups at the start. At the age of 8 months, the mean titer of anti-dsDNA antibodies in the FTY720 treated mice decreased significantly in comparison with controls. The extent of the decrease in the levels

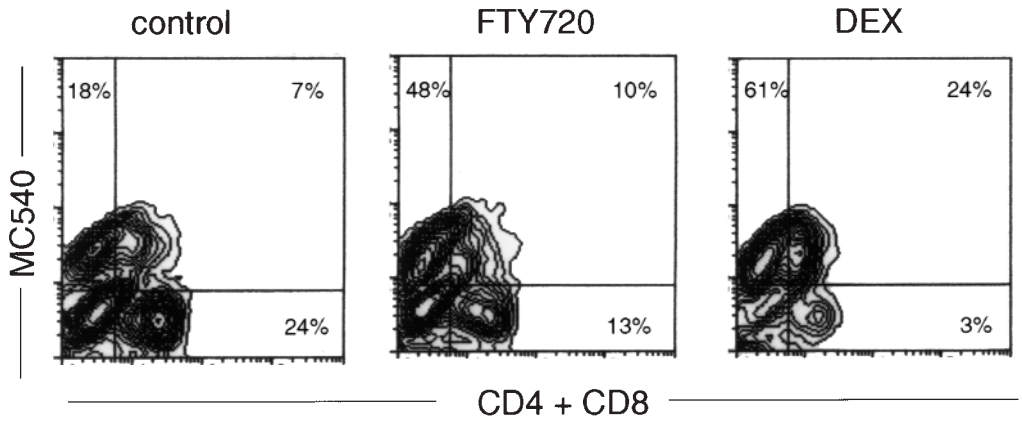

Figure 3. Identification of apoptotic cells *in vitro* in single positive or double negative splenic T cells from 6-month-old MRL/lpr mice. Splenic T cells from female 6-month-old MRL/lpr mice were cultured 12 h in the absence (untreated) or presence of 2 µM FTY720 or 2 µM DEX. The cells were recovered, stained with a combination of FITC conjugated anti-CD4 and anti-CD8 Mab, as well as MC540, and analyzed by 2 color flow cytometry. Percentage of apoptotic cells was determined as MC540^{high} in double negative or single positive T cells. Representative results of 5 experiments are shown here.

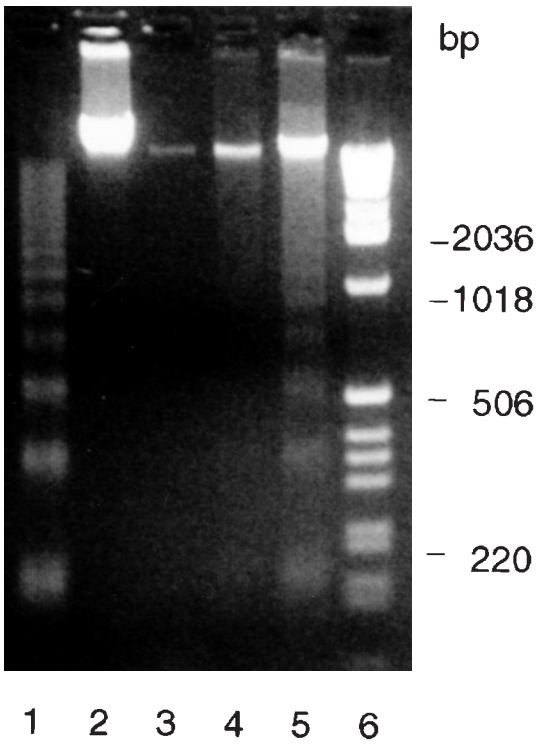

Figure 4. Agarose gel analysis of DNA extracted from splenic T cells from MRL/lpr mice cultured for 4 h. Splenic T cells were cultured 4 h and DNA was extracted and analyzed by gel electrophoresis. Lane 1: Splenic T cells from MRL/lpr mice cultured with 2 µM dexamethasone. Lane 2: Splenic T cells from MRL/+ mice cultured with medium alone. Lane 3: Splenic T cells from MRL/+ mice cultured with 2 µM FTY720. Lane 4: Splenic T cells from MRL/lpr mice cultured with medium alone. Lane 5: MRL/lpr mice cultured with 2 µM FTY720. Lane 6: Control DNA digested with *Hinf I*.

of anti-dsDNA antibodies, however, was higher with mPSL than FTY720 (Figure 7).

Lymphoid proliferation and cell populations in the spleen. In a parallel experiment, 16-week-old female MRL/lpr mice were treated with FTY720, mPSL, or vehicle alone for 16 weeks and then examined. Six control animals and one mPSL treated mouse died before the completion of the experiments, probably due to renal failure, and therefore were excluded from further analyses. There were significant reductions in weight of the cervical lymph nodes and spleen, as well as in the cell number in the spleen from FTY720 and mPSL treated mice, in comparison with controls (Table 1). Spleen cells freshly isolated from MRL/lpr mice were subjected to FACScan analysis. As shown in Figure 8, oral FTY720 markedly reduced the frequency of double negative T cells to an extent comparable with that of mPSL. Table 1 summarizes changes in frequency and number of T, B, and myeloid cells in the spleen and bone marrow. Both frequency and number of double negative T cells in the spleen were significantly decreased by FTY720. Although the frequency of B cells was unchanged among the groups, the total number of B cells in the spleen was significantly reduced in both FTY720 and mPSL treated mice in comparison with controls. Since FTY720 increased apoptosis in the bone marrow cells *in vitro*, the effects of FTY720 on the bone marrow *in vivo* were examined. The number of Gr-1+ myeloid cells in the bone marrow was not significantly reduced in the FTY720 treated mice compared to controls.

Proliferation of spleen cells and secretion of IL-2 in vitro. FTY720 significantly increased the responsiveness of splenic T cells to the stimulation with anti-CD3 (5 mg/ml) in comparison with control mice (Figure 9A). This increase in responsiveness by FTY720 reached almost the same level as that of splenic T cells from MRL/+ mice stimulated with anti-CD3 antibody. The ability to produce IL-2, in response to stimulation with anti-CD3, increased significantly in the FTY720 treated mice, in parallel with an increase in T cell proliferative responsiveness (Figure 9B).

Table 1. Effects of FTY720 and methylprednisolone (mPSL) on body weight, lymphoid organ weight, spleen mononuclear cell (MNC) number, and bone marrow cell (BMC) number in female 32-week-old MRL/lpr mice after 16 weeks of treatment.

	Control, n† = 4 (10)	FTY720, n† = 10 (10)	mPSL, n† = 9 (10)
Body Weight, g	35.4 ± 3.1	36.5 ± 4.2	38.6 ± 3.2
Lymph node weight, g	0.41 ± 0.17	0.13 ± 0.12*	0.08 ± 0.02*
Spleen			
Weight, g	1.05 ± 0.34	0.60 ± 0.22*	0.50 ± 0.21*
MNC in spleen (×10^6)	811 ± 263	462 ± 169*	381 ± 160*
B cells, %	5.9 ± 2.6	5.5 ± 1.9	4.1 ± 2.2
B cells in spleen (× 10^6)	47.8 ± 16.5	25.4 ± 8.3*	15.6 ± 7.6*
SP T cells, %	26.4 ± 11.6	75.1 ± 10.9*	71.3 ± 16.0*
SP T cells in spleen (× 10^6)	214 ± 94	346 ± 50	272 ± 61
DN T cells, %	57.9 ± 6.9	13.7 ± 8.2*	16.3 ± 7.9*
DN T cells in spleen (× 10^6)	470 ± 56	63 ± 38*	62 ± 30*
Bone marrow			
BMC (× 10^6)	14.3 ± 3.3	12.8 ± 4.8	14.0 ± 2.3
B cells, %	8.4 ± 2.6	8.1 ± 1.9	7.5 ± 1.1
B cells in BM (×10^6)	1.2 ± 0.4	1.0 ± 0.3	1.1 ± 0.2
Gr–1+ cells, %	68.7 ± 2.9	70.3 ± 2.2	71.8 ± 11.2
Gr–1+ cells in BM (×10^6)	9.8 ± 0.5	9.0 ± 0.3	10.1 ± 1.7

Statistical method: Mann-Whitney U test; * $p < 0.05$. Data are presented as means ± SD. † Number of mice at sacrifice. Figures in parentheses show the number of mice at the start of the experiment. SP: single positive; DN: double negative.

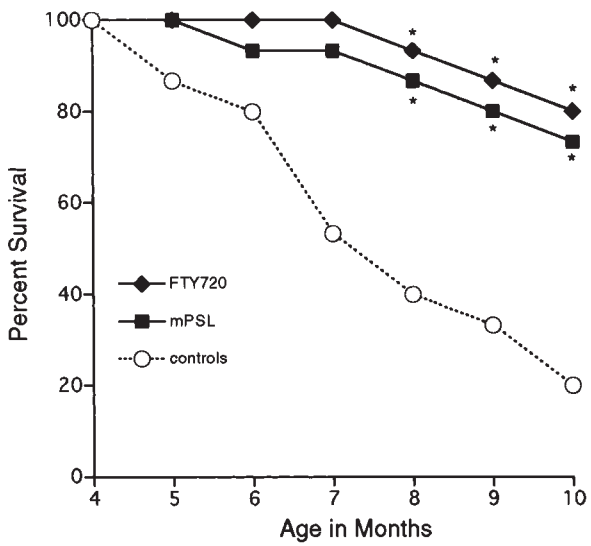

Figure 5. Survival curves for untreated controls, FTY720 treated and mPSL treated female MRL/lpr mice over 10 months. Fifteen MRL/lpr mice at 4 months of age were given *per os* either 2.0 mg/kg of FTY720 or mPSL 3 times a week. Control mice were given 0.5 ml of saline. *Significant difference against untreated controls, $p < 0.01$, chi-square analysis.

DISCUSSION

We have shown that FTY720 is efficacious for the treatment of experimental SLE in MRL/lpr mice. Treatment with FTY720 was tolerated well by MRL/lpr mice with no adverse effects such as bone marrow toxicity (Table 1) or hyperglycemia seen in mPSL treated animals (data not shown). Treatment with FTY720 can reduce adenopathy and splenomegaly, possibly through the induction of lymphocyte apoptosis, and prolong the life span in MRL/lpr mice[21]. Our results have confirmed and extended the capacity of FTY720 for such effects. FTY720 induced apoptosis in double negative T cells *in vitro*, reduced the progression of lupus nephritis in female MRL/lpr mice by inhibiting the development of anti-dsDNA antibodies and decreasing the number of B cells in the spleen, and normalized the proliferation of T cells as well as the production of IL-2 in response to anti-CD3 stimulation by increasing the relative number of functioning T cells.

Several immunomodulating drugs have been evaluated for their therapeutic effects on lupus in MRL/lpr mice. Cyclophosphamide (CYC) was established in 1984 as an effective drug for the treatment of lymphadenopathy, arthritis, and nephritis in MRL/lpr mice[29]. Linomide (LS-2616)[30] and mycophenolate mofetil[31] retard the progression of disease to a degree comparable to that of CYC in MRL/lpr mice. Conflicting results have been published on the therapeutic efficacy of cyclosporin A (CSA). Berden, *et al* did not find any appreciable effects of CSA[32], while Mountz, *et al* described ameliorated immunopathology and prolonged survival with it[33]. Other immunomodulators like FK506[34] and leflunomide[35], when tested in MRL/lpr mice, revealed some favorable effects. Our results indicate a therapeutic efficacy of FTY720 on the disease outcome in MRL/lpr mice.

Recent studies have implicated FTY720 directly in apoptotic cell death of lymphocytes, which may explain its

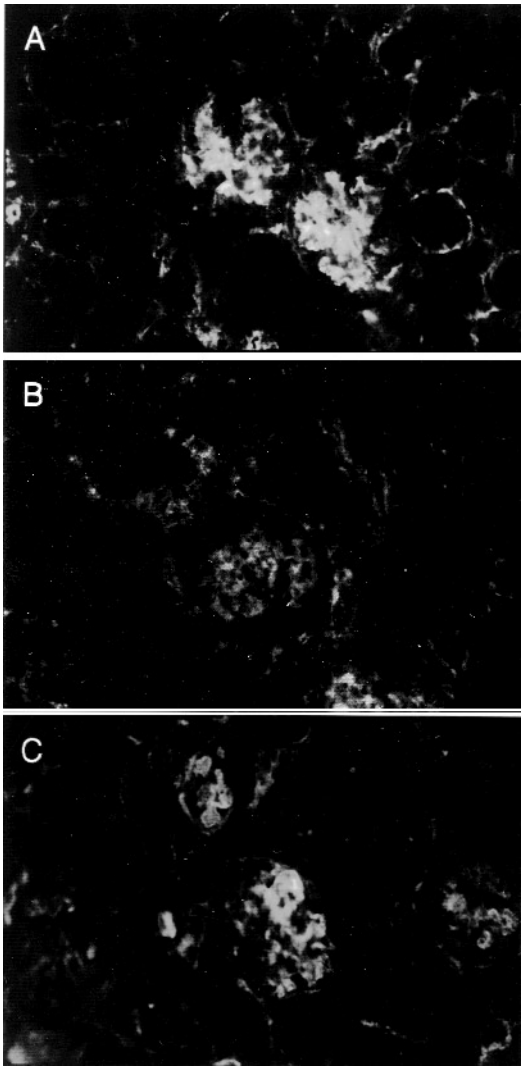

Figure 6. Immunofluorescence stainings of kidney sections from untreated controls (A), FTY720 treated (B) and mPSL treated (C) female MRL/lpr mice. Deposition of IgG in glomeruli from FTY720 treated mice decreased markedly in comparison with untreated controls.

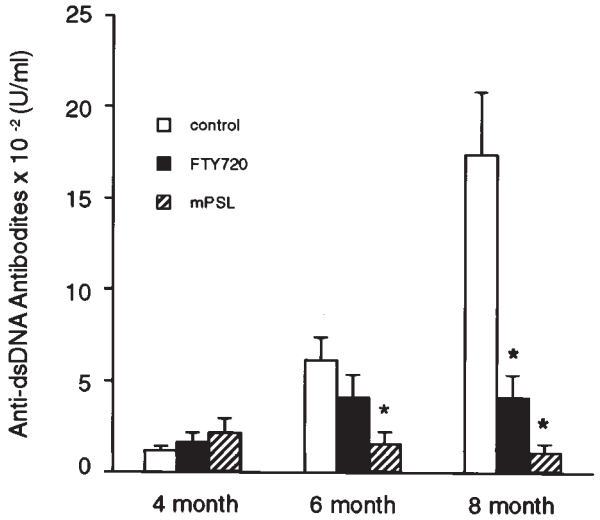

Figure 7. Anti-dsDNA IgG antibodies in untreated controls, FTY720 treated and mPSL treated female MRL/lpr mice. *Titers of anti-dsDNA antibodies significantly higher than those of controls, $p < 0.05$, Mann-Whitney U test.

immunosuppressive effects[21,36,37]. We found that FTY720 induced apoptosis in the thymocytes, but not in the splenic lymphocytes from normal BALB/c mice *in vitro* (Figure 1). Our results have substantial variance with a recent report of the high sensitivity of splenic lymphocytes to FTY720 *in vitro*[38]. The reason for this discrepancy is unclear; it may be ascribed to the different concentrations of FTY720 used in their studies and ours. Our experiments were performed with 2 µM, which was found to be the lowest concentration for inducing maximal apoptosis in the thymocytes, whereas Shimizu, *et al* applied 10 µM, 5 times higher than we used. We confirmed that FTY720 increased apoptosis in the spleen cells *in vitro* if higher doses (30 µM) were added to culture media, albeit to an extent much lower than that with DEX (data not shown). However, a dose-response curve needs to be drawn to delineate precisely the cell selectivity of FTY720. Unlike in normal mice, FTY720 at 2 µM increased apoptosis in splenic double negative T cells from MRL/lpr mice in a relatively specific manner, whereas DEX at the same concentration induced apoptosis in both double negative and single positive T cells (Figures 2, 3, and 4). Although the reason for this apparent difference between FTY720 and DEX is yet to be determined, there are several possible explanations. First, the machinery of apoptosis triggered by FTY720 may be quite different from that of DEX. A recent study indicated that apoptosis induced by FTY720 involves the activation of c-Jun NH_2-terminal kinase and p38 pathways[16]. Second, it is also possible that FTY720 could be intrinsically weaker as a stimulus than DEX; the sensitivity of double negative T cells to apoptosis appears to be higher than that of single positive T cells from MRL/lpr mice[39]. Third, FTY720 may selectively affect the lymphocytes in division and/or activation. Several studies have shown that double negative T cells in MRL/lpr mice are rapidly cycling in a state of activation[40,41]. Moreover, FTY720 has been reported to increase activation induced peripheral T cell death in which Fas/Fas-Ligand (Fas-L) system is involved[38]. Of particular interest are the recent observations that intracellular signaling of the Fas receptor is coupled to the sphingomyelin-ceramide pathway, which induces apoptosis[42]. It is tempting to speculate that apoptosis induced by FTY720 may be mediated through a sphingomyelin-ceramide cycle by means of bypassing the Fas/Fas-L interaction, because FTY720 resembles sphingosine in chemical structure[8]. Preliminary experiments suggested that the mechanism of apoptosis induced by FTY720 *in vitro* is similar to that of the membrane permeable ceramide analog, C2-ceramide, which also suppresses autoimmunity and mortality in MRL/lpr mice (Okazaki H, *et al*, manuscript in preparation). Consistent with our obser-

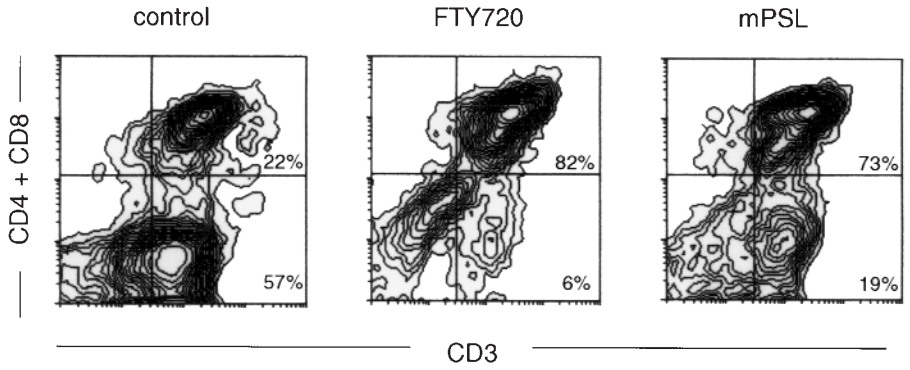

Figure 8. Phenotypic analysis of spleen cells from untreated controls, FTY720 and mPSL treated MRL/lpr mice at age 32 weeks. Freshly isolated spleen cells were analyzed by 2 color staining. *X* and *Y* axes represent FITC and PE, respectively. For each analysis, 5×10^4 cells were used. Representative results of 4 separate experiments are shown.

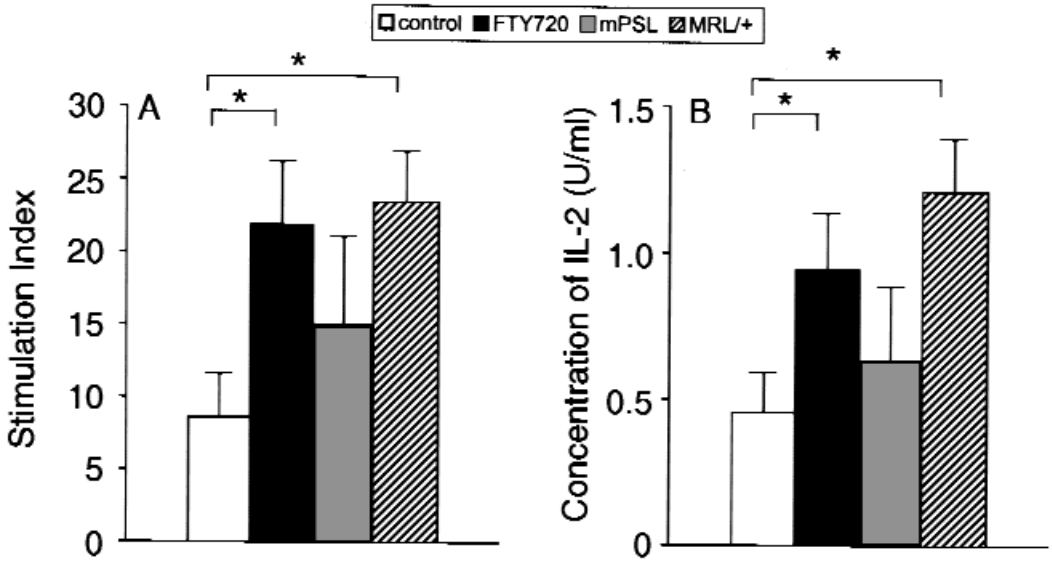

Figure 9. Spleen cell proliferation and IL-2 secretion *in vitro* in untreated controls, FTY720 treated and mPSL treated female MRL/lpr mice, and age matched MRL/+ mice. A. Proliferative responses of splenic T cells stimulated by immobilized anti-CD3 antibodies. Results are expressed in the stimulation index. B. Concentrations of IL-2 (U/ml) after stimulation with anti-CD3. *Significant differences between FTY720 or mPSL and controls, $p < 0.05$, Mann-Whitney U test.

vations *in vitro*, FTY720 markedly decreased the number of double negative T cells in the periphery (Figure 8), resulting in a reduction in adenopathy and splenomegaly. Moreover, FTY720 significantly increased T cell proliferation and IL-2 production to the stimulation with anti-CD3, while mPSL did not (Figure 9). There were no differences between mice treated with FTY720 and mPSL in the degrees of reduction in double negative T cells, which are considered anergic to activation stimulus[20] (Figure 8). Thus, it is possible that glucocorticoids exert immunosuppressive effects on the whole array of T cells nonspecifically, while FTY720 preferentially induces apoptosis in abnormally propagated T cells in MRL/lpr mice. Although FTY720 induced apoptosis in the double negative T cells in MRL/lpr mice *in vitro*, there is no direct evidence of FTY720 induced apoptosis in lymphoid organs *in vivo* from this study. In this regard, Nagahara, *et al*[43] reported that FTY720 induced apoptosis *in vivo* in circulating T cells in rats and mice. The contribution of the FTY720 induced apoptosis on the therapeutic effect *in vivo* remains to be determined.

It is necessary to clarify the additional immunosuppressive effects of FTY720 other than the apoptosis-inducing activity. We did not examine the effects of FTY720 on the thymoctes from MRL/lpr mice *in vivo*. Recently, Yagi, *et al*[44] reported that *in vivo* administration of FTY720 did not decrease the number of thymocytes from normal mice by

inhibiting the export of mature thymocytes to the periphery. Since double negative T cells in MRL/lpr mice could be derived from double positive cortical thymocytes[45], a decrease in double negative T cells in the periphery might be due to the inhibitory effect on T cell migration from the thymus. Moreover, FTY720 induced apoptosis of the bone marrow cells *in vitro*, while it did not *in vivo*. One possibility is that the concentration of FTY720 in the bone marrow may not have reached the level used *in vitro*. A second possibility is that the same mechanism might be operating in the bone marrow by inhibiting the cellular export from the bone marrow to the periphery.

The main cause of death in MRL/lpr mice is renal failure, and the significantly prolonged survival we found with FTY720 was presumably due to the reduced progression of glomerulonephritis. The observation that FTY720 decreased the deposition of IgG in the glomeruli in mice that had already developed renal disease indicates a therapeutic potential of FTY720 on established renal diseases. The mechanism by which FTY720 ameliorates glomerulonephritis is not fully understood. Recently, by establishing class I or II MHC deficient MRL/lpr mouse strains, it has been revealed that lymphoproliferation of double negative T cells and lupus nephritis in MRL/lpr mice were induced by distinct mechanisms[46,47]. Clearly, a decrease in the number of splenic B cells for a reduced production of pathogenic IgG anti-dsDNA antibodies is among the major factors of such effects. Moreover, a recent study showed that FTY720 decreases the infiltration of T cells into skin allografts in rats[48]. A reduced T cell influx into kidneys with glomerulonephritis could limit the local production of disease-promoting cytokines.

We have shown therapeutic effects of FTY720, a novel apoptosis-inducing drug, in MRL/lpr mice. Probably due to its selective influence on activated T cells, FTY720 had the merit of few, if any, serious side effects in murine experimental SLE. Based on these results, we propose that FTY720 warrants clinical trials as a potential modifier of autoimmune diseases in humans.

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