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Experimental treatment of autoimmune MRL-lpr/lpr mice with immunosuppressive compound FK506

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SUMMARY

A newly developed immunosuppressive drug, FK506 (Fujisawa, Japan) is known to inhibit T-cell immunity. We have evaluated the action of this compound in MRL/lpr mice which develop a severe autoimmune disease. Eight-week-old female MRL/lpr of mice were treated subcutaneously with 2 mg/kg (high dose), 0·8 mg/kg (medium dose), 0·2 mg/kg (low dose) or solvent only (control) six times per week. Survival times of the mice were prolonged in the medium and the high dose treatment groups. The lymph node swelling was dramatically prevented with the high dose treatment. The increasing footpad swelling seemed to be also suppressed with the treatment. FACS analyses of the spleen cells revealed that FK506 reduced the percentage of double negative T cells (Thy-1.2$^+$, Lyt-2$^-$, L3T4$^-$). Serological studies showed that anti-ssDNA and anti-dsDNA activities were significantly reduced by the high dose treatment, which is different from recent findings with Cyclosporine A. The high dose treatment also suppressed the total amount of IgG, even though the IgG concentration was rather increased by the medium dose treatment. Decreased proteinuria as well as pathological evaluations of the kidneys and lungs indicated that there were marked ameliorations in these organs with the treatment. These results suggest that FK506 could be potentially used for the treatment of autoimmune diseases.

INTRODUCTION

MRL-lpr/lpr mice develop a spontaneous and aggressive autoimmune disease, which resembles human systemic lupus erythematosus (SLE). The characters of the generalized autoimmune disease include marked lymphadenopathy, auto-antibody production, lupus nephritis, interstitial pneumonitis, arthritis and premature death (Murphy & Roth, 1977; Andrew *et al.*, 1978). Neonatal thymectomy had a significant suppressive effect on the disorder (Steinberg *et al.*, 1980; Theofilopoulos *et al.*, 1981) and the lymphadenopathy consists primarily of the abnormal proliferation of T cells with a unique phenotype (Morse *et al.*, 1982). Thus, it is generally believed that T lymphocytes play an important role in the pathogenesis. However, other factors are involved in the development of this complex type of autoimmune disease (Davidson *et al.*, 1984).

Several immunosuppressive drugs, including Cyclosporine A, have been used to treat MRL-lpr/lpr mice (Smith, Chused & Steinberg, 1984; Isenberg *et al.*, 1981; Okudaira *et al.*, 1986; Mountz *et al.*, 1987). The results of these experiments have contributed to understanding the more precise mechanisms in the autoimmune phenomenon of the mouse. In the present study, we applied the newly developed immunosuppressive compound, FK506 (Kino *et al.*, 1987a, b; Thomson, 1989), which is known to inhibit especially T-cell immunity, to the experimental treatment of MRL-lpr/lpr mice. We found that FK506 had strong immunosuppressive effects on several aspects of the autoimmune disease.

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MATERIALS AND METHODS

Mice

MRL-lpr/lpr mice (breeding pairs provided by Dr Murphy, the Jackson Laboratory, Bar Harbor, ME) were bred in the Central Institute for Experimental Animals (Kanagawa, Japan). The animals were specific pathogen free.

FK506 administration and the experimental protocols

FK506 (Fujisawa Pharm. Co. Ltd, Osaka, Japan) dissolved in carrier solvent (HCO-60 and D-mannitol) was diluted in phosphate-buffered saline (PBS). Eight-week-old female MRL-lpr/lpr mice were administered subcutaneously six times a week. For control, mice were given the equivalent dose of carrier solvent and PBS. For dose–response analyses, 2 mg/kg/day as a high dose, 0·8 mg/kg/day as a medium dose, and 0·2 mg/kg/day as a low dose treatments were used. Other experiments were

carried out with the high dose treatment and the placebo injection. The adenopathy was evaluated as scores. The diameter of each palpable lymph node was measured and graded as follows: 0, 0–5 mm; 1, 6–10 mm; 2, 11–15 mm; 3, 16–20 mm; 4, 21–25 mm. The total score of each mouse was the summation of the individual scores of the lymph nodes. Proteinuria was determined semi-quantitatively by colorimetric reaction with Combistix paper (Sankyo. Tokyo, Japan). The measurement of the hind footpads was performed with a dial thickness gauge (Ozaki MFG. Tokyo, Japan).

Histological evaluation

Kidneys and lungs were removed, fixed in 10% formalin and processed according to standard techniques. These sections were stained with hematoxylin and eosin.

Serological analyses

Sera were assayed for anti-single stranded (ss) DNA or anti-double stranded (ds) DNA activities of IgG and IgM classes by enzyme-linked immunosorbent assay (ELISA) as described (Okudaira *et al.*, 1981). In brief, the denatured ssDNA was pepared by boiling a solution of calf thymus DNA (10 μg/ml, type I; Sigma, St Louis, MO). The dsDNA was produced by S1 nuclease treatment (Takara Shuzo, Kyoto, Japan). The DNA was bound to the wells of microtitre plates which were pretreated with poly-L-lysin (Sigma). The plates were blocked with 1% bovine serum albumin (Sigma) plus 0·5% bovine gamma globulin and serial diluted test sera were incubated. After washing peroxidase-conjugated goat anti-mouse IgM, or anti-mouse IgG antibodies (Cappel, U.S.A.) were added. The activity was measured by *O*-phenylene diamine solution (0·4 mg/ml) and Titertex Multiskan. A mixed sera from old MRL-lpr/lpr mice was used for a standard and 1000 units were the equivalent of each activity in this serum. The measurement of total immunoglobulin concentrations was performed by radial immunodiffusion plates (The Binding Site, Birmingham, U.K.)

Cell staining and flow cytometry

Single cell suspensions of splenocytes were incubated with fluorescinated monoclonal anti-Thy-1.2, anti-Lyt-2, anti-L3T4 (Beckon-Dickinson, U.S.A.) or anti-B220 (kindly supplied by Dr K. Okumura, Juntendo University) antibodies and were analysed by a fluorescence-activated cell sorter (FACS).

Statistical analysis

Statistical significance of differences was analysed by Student's *t*-test or Wilcoxon rank test.

RESULTS

Effect of FK506 on the survival time of MRL-lpr/lpr mice

In order to evaluate the effect of FK506, subcutaneous injection (6 times a week) was started on 8-week-old female MRL-lpr/lpr mice using high dose (2 mg/kg/day, 8 mice), medium dose (0·8 mg/kg/day, 7 mice), low dose (0·2 mg/kg/day, 8 mice) or carrier solvent (for control, 9 mice). As shown in Fig. 1, half of the control mice died prior to 25 weeks old. On the other hand, nearly all mice in the high and medium dose groups survived to that age. Thus it was concluded the high dose and the medium dose therapy significantly prolonged the survival time of

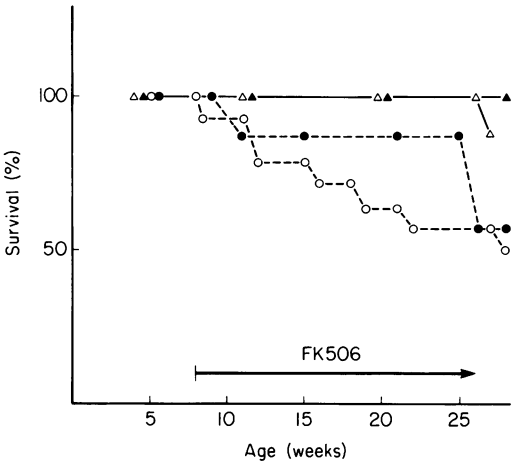

Figure 1. Effect of FK506 on survival of female MRL-lpr/lpr mice. Mice of each group (8–9 mice) were treated with subcutaneous injection 6 times per week (—▲— FK506 2 mg/kg; —△— FK506 0·8 mg/kg; --●-- FK506 0·2 mg/kg; --○-- control).

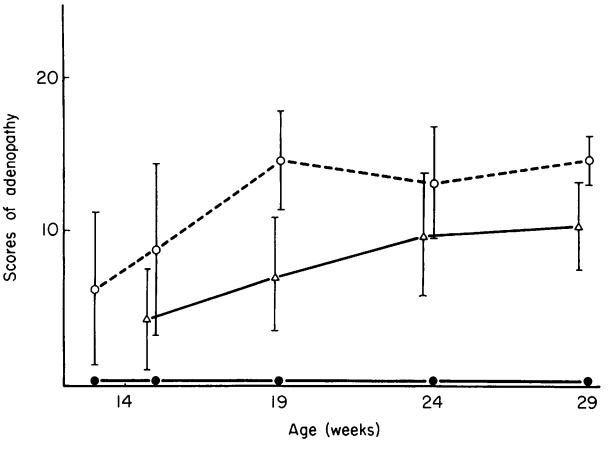

Figure 2. Effect of FK506 on the lymphadenopathy of MRL-lpr/lpr mice (each group consisted of 8–9 mice). The adenopathy was evaluated as scores as described in the Material and Methods (--○-- control; —△— FK506 0·8 mg/kg; —●— FK 506 2 mg/kg).

MRL-lpr/lpr mice compared to the control treatment. The low dose treatment also seemed to have some effect on the survival. At the age of 25 weeks, body weight of mice in each group was as follows: control group, 43·2 ± 3·4 g; low dose group, 43·4 ± 4·9 g; medium dose group, 39·8 ± 2·6 g; high dose group, 36·7 ± 3·2 g.

Effect of FK506 on the lymphadenopathy

The lymphadenopathy was evaluated by scores as described in the Materials and Methods. The placebo-treated mice gradually developed the lymph node swelling (Fig. 2). The high dose treatment of FK506 completely prevented this hyperplasia. Mice treated with the medium dose showed intermediate size of lymph nodes between those of the control and the high dose treatment. The low dose treatment did not have a significant effect on the lymphadenopathy (data not shown).

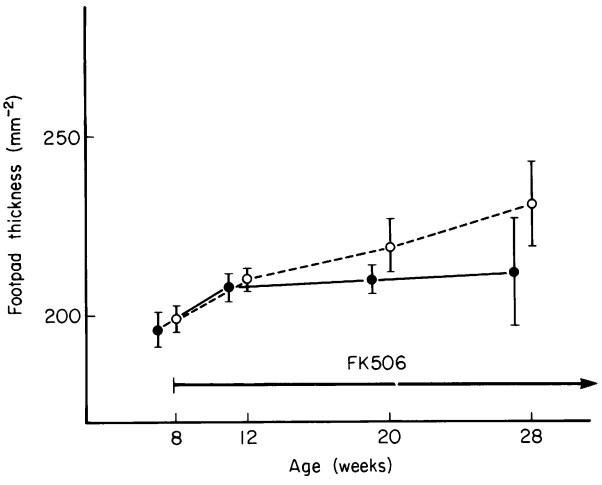

Figure 3. Effect of FK506 on the hind footpad swelling of MRL-lpr/lpr mice (5 mice in each group) (—o— control; —●— FK506 2 mg/kg).

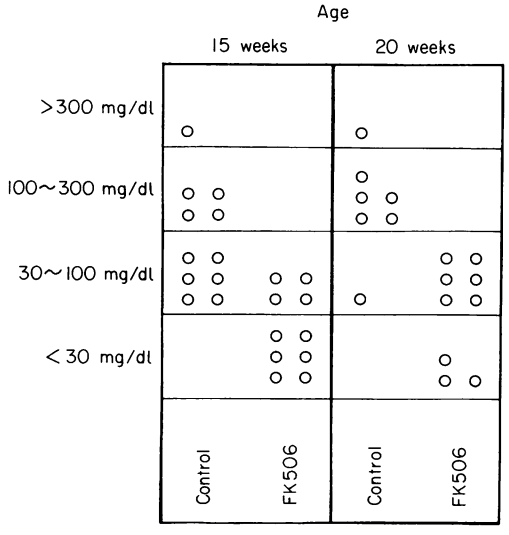

Figure 4. Effect of FK506 on proteinuria. Proteinuria was determined semiquantitatively by colorimetric reaction.

Arthritis

MRL-lpr/lpr mice are also known to develop spontaneous arthritis (Hang, Theofilopoulos & Dixson, 1982). For the evaluation of the effect of FK506 on the arthritis, the thickness of the hind footpad was measured (Fig. 3). FK506 high dose treatment significantly suppressed the swelling of the hind footpad at the age of 28 weeks ($Po\{W \geqq 38\} = 0{\cdot}016$, Wilcoxon rank test).

Effect of FK506 on lupus nephritis and interstitial pneumonitis

Proteinuria was semi-aquantitatively estimated. The high dose treatment suppressed the amounts of proteinuria (Fig. 4). This finding was also confirmed by the histological analysis. Mice treated with high dose FK506 (2 mg/kg/day, 9 mice) and mice of placebo treatment (9 mice) were killed at the age of 4–6 months (3 mice every month) and the histological changes were compared (Table 1). Figure 5 shows the representative pictures. In the kidney of the control mice, there were marked cellular infiltrations around arteries and the arterial walls were thickened. The glomerulus were enlarged and lobulated. Hypercellularity in the glomerulus was also recognized. On the contrary the kidneys of FK506-treated mice were found to be nearly normal.

MRL-lpr/lpr mice also develop spontaneous interstitial pneumonitis (Okudaira *et al.*, 1986). The thickening of alveolar septa and the infiltration of mononuclear cells were observed in the lung of control mice. FK506 clearly suppressed the development of this pneumonitis (Table 1, Fig. 5).

Serological analysis

Sera were collected individually and the anti-ssDNA and anti-dsDNA activities of IgM and IgG classes of each serum were evaluated by ELISA. Control mice gradually develop IgG class of anti-ssDNA and anti-dsDNA activities and IgM class of anti-dsDNA (Table 2). On the contrary, FK506 high dose treatment significantly reduced the activities. The medium dose treatment also seemed to have suppressive effects on the most of anti-DNA activities, even though no statistical significance was obtained compared to the control. Next, total IgG were evaluated by the single radial diffusion plates. The high dose treatment reduced the IgG concentration. Interestingly, the medium dose treatment rather enhanced the IgG concentration (Table 3).

Changes of spleen cell phenotypes with FK506 treatment

FK506 dramatically suppressed the hyperplasia of lymph nodes. Thus, it was rather difficult to obtain a proper amount of lymph node cells from mice treated with FK506. In accordance with the effect on the lymph node swelling, the weight of spleens was significantly reduced by the FK506 treatment (For example at the age of 6 months, control mice had 800 ± 350 mg of spleen and FK506 mice possessed 250 ± 120 mg of spleen). In order to investigate the effect of the treatment on the different cellular populations, 6-months-old mice were killed and the single cell suspensions from spleen were analysed with monoclonal antibodies and FACS. Control mice exhibited a high percentage ($42{\cdot}0 \pm 8{\cdot}5$) of double negative T cells (Thy-1.2$^+$, Lyt-2$^-$, L3T4$^-$), which is characteristic for this mouse. FK506 predominantly suppressed the percentage of this abnormal cell population (Table 4).

DISCUSSION

FK506, a newly developed immunosuppressive compound, had significant effects on the survival time, lymphadenopathy, autoantibodies, nephritis, arthritis, interstitial pneumonitis and the abnormal proliferation of the double negative T cells of MRL-lpr/lpr mice. These results suggest that this chemical could potentially be used for the treatment of human autoimmune diseases. It was also shown that FK506 had a significant immunosuppressive effect on allograft rejection (Ochiai *et al.*, 1987; Inamura *et al.*,1988a) and collagen-induced arthritis (Inamura *et al.*, 1988b) in rats.

The similar immunosuppressive effects on MRL-lpr/lpr mice have been reported with Cyclosporin A (Smith, Chused &

Table 1. Histological evaluation of kidney and lung from FK506-treated MRL-lpr/lpr mice

Age	4 months		5 months		6 months	
Group*	Control	FK506	Control	FK506	Control	FK506
Kidney						
Cell numbers in one glomerulus	69±10	39±8†	62±15	40±7†	88±32	50±17†
Perivascular infiltration	++‡	−~±	++~+++	−~+	+++	−~+
Extra capillary crescent	+	−	++	−	++~+++	−
Lung						
Thickening of alveolar septae	−	−	++	+	++	+
Infiltration of mononuclear cells in alveolar septae	+	−	++	+	++	+
Peribronchial and perivascular lymph follicles	−	−	+	−	++	+
Germinal centers	−	−	+	−	+	−

*Each group consisted of three mice. FK506 dose was 2 mg/kg per day.
†$P < 0.005$.
‡Arbitrary scale of histological findings: −, not observed; +, mild; ++, moderate; +++, severe changes.

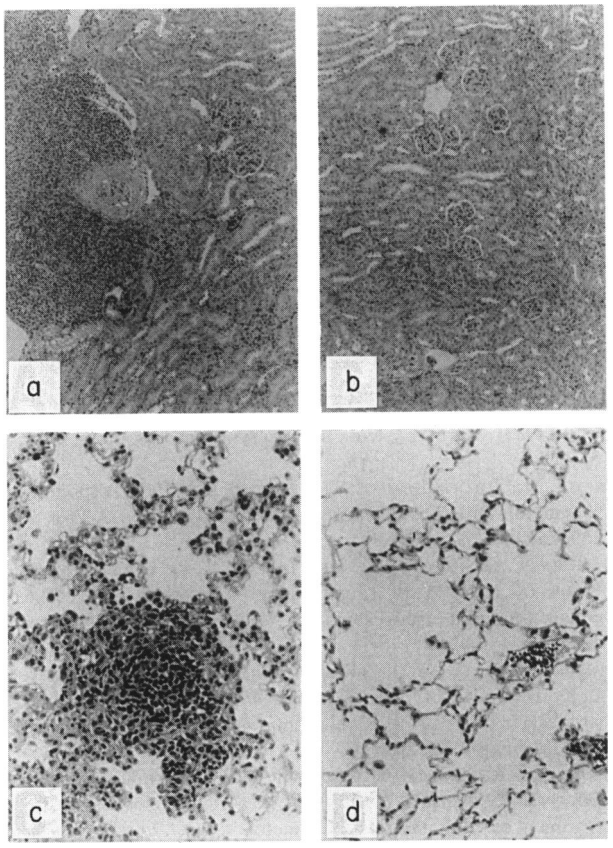

Figure 5. Representative kidney and lung sections from FK506 or placebo-treated MRL-lpr/lpr mice. (a) Kidney from placebo-treated 6-month-old mice (×65). (b) Kidney from FK506 (2 mg/kg/day)-treated 6-month-old mice (×130). (c) Lung from placebo-treated 5-month-old mice (×130). (d) Lung from FK506 (2 mg/kg/day)-treated 5-month-old mice (×130).

Table 2. Effect of FK506 on anti-DNA antibodies in MRL-lpr/lpr mice

Age	Group†	Anti-ssDNA (units)*		Anti-dsDNA (units)*	
		IgM	IgG	IgM	IgG
3 months	Control	660±364	314±216	32±11	51±10
	Medium dose**	244±213	ND‡	70±57	ND
	High dose**	86±21§	8±4§	18±20	13±5¶
6 months	Control	369±111	476±229	363±132	362±243
	Medium dose	353±90	294±274	218±139	175±169
	High dose	117±55¶	37±35§	9±5¶	6±1§

*One thousand units are the anti-DNA activity of each class in the pooled standard serum from old MRL-lpr/lpr mice.
†Each group consisted of six or seven samples
‡ND, not determined; §$P < 0.01$; ¶$P < 0.005$.
**High dose was 2 mg/kg/day; medium dose was 0·8 mg/kg/day.

Table 3. Effect of FK506 on serum IgG concentrations (mg/ml) in MRL-lpr/lpr mice*

Age	5 months	7 months
Control	13·7±2·8	17·7±6·8
Low dose†	17·3±1·5	ND‡
Medium dose†	29·0±2·8§	33·6±7·4**
High dose†	5·2±2·4¶	4·8±0·5¶

*Four to six mice per group were tested individually by radial immunodiffusion plates.
†High dose was 2 mg/kg; medium dose was 0·8 mg/kg; low dose was 0·2 mg/kg.
‡ND, not determined; §$P < 0.005$; ¶$P < 0.01$; **$P < 0.05$.

Table 4. Surface phenotypes of spleen cells from FK506 treated MRL-lpr/lpr mice*

Antigen	B220$^+$	Thy-1.2$^+$	Lyt-2$^+$	L3T4$^+$	Thy-1.2$^+$, Lyt·2$^-$, L3T4$^-$
Control	55·2±1·3	68·7±8·0	8·5±1·9	18·2±3·6	42·0±8·5
FK506†	49·3±2·7	40·4±12·5	10·7±2·4	20·6±5·5	9·1±4·7‡

*Six-month-old mice (3 mice in each group) were killed and the spleen cell suspensions were analysed by FACS after staining with FITC-conjugated monoclonal antibodies. Values were positive percentage of the stained cells.

†The dose was 2 mg/kg/day.

‡$P<0·01$.

Steinberg, 1984; Isenberg *et al.*, 1981; Okudaira *et al.*, 1986; Mountz *et al.*, 1987). However, there are some differences. firstly, the dose of FK506 was $\frac{1}{10}$ to $\frac{1}{20}$ lower than that of Cyclosporin A. Secondly, our data clearly demonstrated that FK506 suppressed anti-ssDNA and dsDNA antibodies. On the other hand, recent data with Cyclosporin A showed that the anti-DNA antibodies were not affected by the treatment (Mountz *et al.*, 1987). Therefore, Mountz *et al.* pointed out, anti-DNA antibodies might have no relation to the immunopathology because Cyclosporin A nevertheless decreased the pathological changes in arthritis and glomerulonephritis. However, there is ample evidence supporting the relationship of anti-DNA antibodies and lupus nephritis (Borel *et al.*, 1978; Dixon *et al.*, 1983; Yoshida *et al.*, 1981; Brunea & Benveniste, 1979). In this regard, FK506 could be more effective on these autoimmune mice because it suppressed T-cell and B-cell immunity. The difference of IgG concentration in the sera from the high dose treated and the medium dose treated mice is interesting. Although the reduced IgG with the high dose treatment is clear, the elevated IgG with the medium dose treatment could be in part affected by the reduced rheumatoid factor by the treatment. This needs further investigation.

There were some differences in the body weight of mice between the control group and high dose group. It is known that MRL-lpr/lpr mice abnormally gain weight perhaps due to the hyperplasia in the lymphoid organs, edema and some other mechanisms. Thus, it is possible to consider this body weight loss as a therapeutic effect of FK506. In fact, the high dose treated mice looked to be very healthy and active compared to the control mice at the same age. On the contrary, it is also possible that this body weight loss could be due to a side-effect (Inamura *et al.*, 1988b). Recent experiments suggested that low calorie intake prolonged the life span of MRL-lpr/lpr mice and reduced lympho-proliferation (Beach, Gershwin & Hurley, 1982; Kubo, Day & Good, 1984). However, it is extremely unlikely that the effects of FK506 on MRL/lpr mice could only be due to the food intake restriction. For example, calorie intake did not significantly reduce anti-DNA antibodies. Further studies should be carried out in order to know the possibilities of the side effects. However, at the present time, FK506 seems to be a quite promising drug for the treatment of autoimmune diseases.

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