Studies on *Lysimachia hemsleyana* MAXIM. II. Effect of the drug on autoimmune MRL/Mp-lpr/lpr mice

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Abstract

MRL/Mp-lpr/lpr (MRL/l) mice, the murine model of systemic lupus erythematosus (SLE), were treated *per os (p.o.)* with *Lysimachia hemsleyana* MAXIM. (LhM). The effects with such treatment were ; i) reduction of characteristic lymphadenopathy, ii) decrease in serum anti-dsDNA antibody, iii) improvement of interleukin-2 (IL-2) production by the stimulation of spleen cells with concanavalin A (Con A). These results suggest that this drug is useful to treat SLE.

Key words *Lysimachia hemsleyana* MAXIM., MRL/Mp-lpr/lpr, autoimmunity, autoantibody, mixed lymphocyte reaction, interleukin 2.

Abbreviations BSA, bovine serum albumin ; C3H, C3H/He ; Con A, concanavalin A ; HBSS, Hanks' balanced salt solution ; IL-2, interleukin 2 ; LhM, *Lysimachia hemsleyana* MAXIM. (Jin-Qian-Cao), 金銭草 ; LPS, lipopolysaccharide ; MLR, mixed lymphocyte reaction ; MMC, mitomycin c ; MRL/l, MRL/Mp-lpr/lpr ; PBS, phosphate buffered saline ; PHA, phytohemagglutinin ; *p.o.* ; *per os* ; SI, stimulation index ; SLE, systemic lupus erythematosus

Introduction

MRL/Mp-lpr/lpr (MRL/l) mice develop a fulminant autoimmune disease accompanied by a marked lymphadenopathy, hypergammaglobulinemia and autoantibody production.[1,2] The lymphadenopathy consists primarily of T cells with an unusual phenotype. These T cells have defects in production of and response to interleukin-2 (IL-2).[3] The generalized autoimmune disease of these mice affects the joint, kidney, lung, and blood vessel.[1,2] Pathological findings in their kidneys consist of cellular infiltration and sclerosis of glomeruli as well as interstitial inflammation.[1,2] Infiltration of lymphocytes become evident in various organs by 4 months of age, coinciding with the time of marked lymphadenopathy. Therefore, MRL/l mice may offer an excellent model to study therapeutic regimens possibly applicable to human disease.

Lysimachia hemsleyana MAXIM. (LhM) has an immunosuppressive effect on the induction of the cell-mediated and humoral immune responses, besides this agent has no direct side effects.[4-6]

In the present study, we found that LhM has a therapeutic effect on autoimmune-prone MRL/l mice reducing lymphadenopathy, decreasing anti-dsDNA antibody and improving IL-2 production.

Materials and Methods

Mice : MRL/Mp-lpr/lpr (MRL/l, H-2^k) mice were originally obtained from the Seiwa Laboratory Animal Institute (Fukuoka, Japan) and bred in our own animal facility. BALB/c (H-2^d), CBA/J (H-2^k), and C3H/He Slc (C3H, H-2^k) mice were purchased from the Shizuoka Laboratory Animal

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Extraction of Lysimachia hemsleyana MAXIM. : The roots of *Lysimachia hemsleyana* MAXIM. produced in China, were finely powdered and extracted with boiling distilled water for 60 min (roots : water = 1 : 10, w/v), as described previously.[6] Extract of LhM was dissolved in phosphate-buffered saline (PBS) and after keeping in a boiling water bath for 10 min and cooling at a concentration of 100 mg/ml, 1500 mg/kg of LhM extract was given *per os (p.o.)* to MRL/l mice once a day, every day. Treatment was started at 2 months of age.

Mitogenic stimulation : Mitogenic stimulation was carried out as described previously.[6] Spleen cells were suspended in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% Nuserum (Collaborative Research, Inc., Lexington, MA), 100 U/ml of pencillin, 100 μg/ml of streptomycin and 4×10^{-5} mM 2-mercaptoethanol at a concentration of 2×10^{6} cells/ml and 0.2 ml each of this cell suspension was poured into a 96-well flat bottomed microculture plate (corning 25860). After adding 10 μg/ml concanavalin A (Con A), 100 μg/ml lipopolysaccharide (LPS) or 10 μg/ml phytohemagglutinin-p (PHA-P), which were obtained from Difco Laboratories, Detroit, MI, cells were cultured for 48 hr at 37℃ in a humidified atmosphere of 5% CO_2 in air. One microcurie of [^{3}H]thymidine was added to each well for last 6 hr of cultivation. Cultured cells were then harvested on glass-fiber papers. After drying, the paper disks were placed in a scintillation cocktail, and the incorporated radioactivity was measured in a liquid scintillation counter (Aloka, LSC 703, Tokyo, Japan).

Mixed lymphocyte reaction (MLR) : MLR was carried out as previously described.[7] A mixture of 5×10^{5} responder cells and 5×10^{5} stimulator cells (spleen cells) was cultured in 0.2 ml/well in 96-well flat-bottomed microculture plates. Culture medium as described above was also used for the mitogenic stimulation. Stimulator cells were treated with 40 μg/ml mitomycin C (MMC) for 30 min. Cultures were performed for 96 hr at 37℃ in an atmosphere of 5% CO_2 in air. DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine, as described above. The results are presented according to the net CPM and stimulation index (SI) :

$$SI=\frac{\text{CPM in the presence of MMC-treated allogeneic cells}}{\text{CPM in the presence of MMC-treated syngeneic cells}}$$

Assay for anti-dsDNA and anti-Sm antibodies : Anti-dsDNA and anti-Sm antibodies were titrated using ELISA method according to the method of Fish.[8] Polystyrene laboratory grade plates (Sanko Junyaku, Tokyo, Japan) were coated with 50 μl/well (50 μg/ml) of poly-L-lysine (Sigma Chemical Co., St. Louis, MO) for 1 hr at 37℃. After washing with PBS, 100 μl/well (5 μg/ml) of dsDNA (Sigma, double stranded DNA from calf thymus) was added and incubated overnight at 4℃. In the case of Sm, 100 μg of Sm (Pel-Freeze, 5 μg/ml) was added to polystyrene laboratory grade plates and incubated overnight at 4℃. The plates were washed extensively with PBS containing 0.05% Tween 20. Blocking of the plates with 1% bovine serum albumin in carbonate buffer was carried out for 30 min at room temperature and then the plates were washed again. Sera diluted in 0.05% Tween 20 PBS were added and the preparations were incubated for 120 min at room temperature. After washing the plates with PBS-Tween, 50 μl of a 1 : 1000 dilution of the alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Tago Inc., CA) in carbonate buffer was added to each well and the incubation was continued further for 120 min at room temperature. Thereafter, the plates were washed and incubated with the alkaline phophatase substrate, paranitrophenyl phosphate. Quantitation of antoantibodies was determined using Easy Reader EAR 400 (SLT-Labinstruments, Austria) at 405 nm.

Assay for interleukin-2 (IL-2) activity : 2.5×10^{6} Spleen cells (from 4.5-to 6-months old mice) were cultured in 1.0 ml of the complete medium in the presence of Con A (5 μg) in a 6-well tissue culture plate (Falcon, 3046) for 48 hr at 37℃. The supernatant was collected, filtered, and stored at −20℃ until use for assay. HT-2 cells, an IL-2 dependent T cell line, were used as an indicator for the IL-2 assay. HT-2 cells (5×10^{3} cells/well) were cultured with serially diluted IL-

2-containing supernatants in a flat-bottomed microplate at 37℃ for 24 hr. Six hour before harvesting, 1 μCi [^{3}H]thymidine was added to each well. [^{3}H]Thymidine uptake was measured using a liquid scintillation counter. The IL-2 titer of the supernatants was represented by a half-maximal unit, using a probit analysis. HT-2 cells were kindly provided by Dr. Ross E. Longley, Oklahoma Medical Research Foundation.

Statistical analysis: The data were analyzed using Student's t test and analysis of variance where appropriate. A p value of less than 0.05 was considered to be statistically significant.

Results

Effect of LhM on the lymphoid hyperplasia of MRL/l mice

The most pronounced feature of the lupus-like disease in MRL/l mice is a generalized hyperplasia of the lymphoid tissues. At 3 months of age, the lymph nodes were palpable in both the untreated female and male animals. As the mice aged, the nodes further enlarged and cervical nodes became visible. LhM treatment reduced lymphadenopathy ($p < 0.05$) and splenomegaly ($p < 0.05$) in the MRL/l mice (Table I) compared with untreated mice. The mean cell number in spleens from mice at 4.5−6 months of age was only 1.7×10^8 in the LhM-treated MRL/l mice, while that number in untreated 4.5−6 month-old mice was 5×10^8 ($p < 0.01$). The spleens were reduced in weight, as compared to findings in the untreated group ($p < 0.05$).

Effect of LhM on the proliferative responses to Con A, PHA and LPS

Lymphoid cells from MRL/l mice showed a low ability to respond to the T-cell mitogens, Con A and PHA. LhM treatment did not improve this dysfunction. One and four months after the beginning of LhM treatment (3 and 6 months of age, respectively), response to PHA did not show any change from untreated MRL/l mice (Table II). LhM treatment also did not reduce the LPS-induced B-cell mitogenic response of the spleen cells of MRL/l which are known to be hyper-responsive to LPS.

Effect of LhM on the MLR

Spleen cells from MRL/l (H-2^k) mice at 4 months after LhM treatment showed similar de-

Table I Effect of *Lysimachia hemsleyana* MAXIM. on lymphoid hyperplasia of MRL/l mice.

Treatment	No. of mice	Age (months)	Weight (mg)[a)] lymph node	Weight (mg)[a)] spleen	Total splenic cells (net $\times 10^6$)[a)]
Untreated	9	4.5−6	5622.4±1469.5	862.5±125.1	504.0±246.4
LhM[b)]	7	4.5−6	2550.0±1724.5[c)]	557.8±373.2[c)]	165.7±76.9[d)]

a) Values represent mean±S.D. Lymph node were from jugular, axilla, mesenteric, renal paraaortic and inguinal lymph node.
b) *Per os.*
c) $p < 0.05$ compared with untreated mice.
d) $p < 0.01$ compared with untreated mice.

Table II Mitogen responses in LhM-treated and untreated MRL/l mice.

Age (months)	Mitogen responses (net CPM $\times 10^3$)[a)]							
	medium		Con A		PHA		LPS	
	untreated	LhM[b)]	untreated	LhM	untreated	LhM	untreated	LhM
2	1.8±0.7	1.5±0.4	24.3±4.0	14.1±3.5[c)]	4.2±1.0	4.1±0.3[d)]	22.3±3.7	28.2±7.3[d)]
4.5	3.6±1.3	3.8±1.2	ND[e)]		ND		35.8±1.9	37.4±2.5[d)]
6	4.0±0.7	5.4±0.6	27.8±1.4	9.3±1.0[c)]	12.0±1.6	10.1±1.5[d)]	73.1±4.2	74.2±4.2[d)]

a) The data shown includes 4−6 separate mice in each group. Values represent mean±S.D. of 6 cultures.
b) *Per os.* c) $p < 0.001$ compared with untreated mice. d) Not significant compared with untreated mice.
e) Not determined.

Table III Mixed lympocyte reaction with spleen cells from LhM-treated MRL/l mice.

Responder[a]	Stimulator	[^{3}H]Thymidine incorporation[b]	SI[c]
Untreated-MRL/l	Untreated-MRL/l	4.0±0.3	4.1±0.8
〃	BALB/c	16.5±2.4	
LhM-treated[d]	LhM-treated	4.6±0.3	3.1±0.3[e]
〃	BALB/c	14.1±1.0	
C3H[f]	C3H	3.2±0.4	20.1±10.9
〃	BALB/c	64.6±17.5	

a) Responder (5×10^5) and stimulator (5×10^5) cells were added to each culture. Stimulator cells were treated with mitomycin c before the culture.
b) Values represent mean±S.D. of 5 cultures from 4 mice (net CPM$\times10^3$).
c) Stimulation index. d) *Per os.*
e) Not significant compared with untreated mice.
f) Untreated normal spleen cells were used as responders (positive control).

Table IV Autoantibodies in LhM-treated and untreated MRL/l mice.

Mice	Age (months)	Anti-dsDNA[a] (No. of mice)	Anti-Sm[b] (No. of mice)
CBA/J	4	0.001±0.000 (5)	0.000±0.000 (5)
Untreated-MRL/l	4.5−6	0.238±0.090 (9)	0.052±0.034 (12)
LhM-MRL/l[c]	4.5−6	0.084±0.051 (11)[d]	0.062±0.025 (11)[e]

a) Reciprocal serum dilution is 1600. Assays were carried out at the same time. O.D. at 405 nm, mean±S.D.
b) Reciprocal serum dilution is 800. Assays were carried out at the same time. O.D. at 405 nm, mean±S.D.
c) *Per os.* d) $p<0.01$ compared with untreated mice.
e) Not significant compared with untreated mice.

gree of proliferative responses to allogeneic cells (BALB/c, H-2^d), compared with those from untreated MRL/l mice (Table III).

Effect of LhM treatment on the autoantibody production

As shown in Table IV, sera from untreated mice at 4.5−6 month of age showed prominently high levels of anti-dsDNA antibody. The antibody titres were significantly decreased ($p<0.01$) in the LhM-treated MRL/l compared with those of untreated MRL/l mice.

However, serum anti-Sm antibody titres in the LhM-treated mice were not different from those in untreated mice (Table IV).

Effect of LhM on the production of IL-2

MRL/l spleen cells become defective in IL-2 production with the onset of autoimmune disease. Spleen cells from untreated MRL/l mice produced little IL-2 (mean 1.08±0.36 U/ml, n=9) after stimulation with Con A. On the contrary, spleen cells from LhM-treated MRL/l mice produced high levels of IL-2 (mean 2.34±1.04 U/ml, n=5) compared with those in untreated MRL/l spleen cells ($p<0.01$).

Discussion

In MRL/l mice, a marked splenomegaly and generalized lymphadenopathy occur from 8 weeks of age, and total weight of lymphoid tissue becomes more than 100 times larger compared with that of normal murine strains.[9] The lymphadenopathy consists primarily of T cells with an unusual phenotype. These peripheral T cells are positive for Thy-1 but negative for Lyt-2 and L3T4. In addition, lpr/lpr T cells bear surface markers like B220 and 9F3 which are usually seen on B cells.[10,11] Although the precise mechanisms

of immunological abnormalities in these autoimmune mice are unknown, various hypothesis have been proposed, including intrinsic B-cell defect, T-cell or macrophage abnormalities.[12-15]

T cells of MRL/l strain show a severe defect in IL-2 production and in response to Con A or antigenic stimulation. The defect of IL-2 production in MRL/l mice can be improved by stimulating the spleen cells with the tumor-promoting phorbol ester 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) plus Con A[16] and treatment with cholera toxin.[7]

There are some therapeutic approaches to lupus mice using immunosuppressive agents such as cyclophosphazene-derived drugs and cyclosporin A.[17,18] Prostaglandin E_1 reduces the lymphadenopathy and prolongs the life span of SLE mice.[19] More recently, monoclonal antibodies capable of modulating immune responses have been proved to be available in treating murine SLE. Total lymphoid irradiation[20] and treatment with cholera toxin are also effective.[7]

In the present work, we showed thtat oral administration of LhM suppresses anti-DNA antibody production (Table IV) and reduces lymphadenopathy (Table I). The defect of IL-2 in MRL/l mice can be alleviated by the treatment with LhM. However, LhM did not show any improvement effect on other T cell dysfunctions (mitogen response, MLR, Table II, III), and of B cell function (LPS response, Table II). Strangely, Con A response alone was significantly suppressed, although exact mechanisms are not yet solved. These results may indicate that LhM has an immunosuppressive activity selective for the response to self antigens and can prevent autoimmune disease in MRL/l mice.

Some workers[21] have reported that suppression of B cell function is enough to prevent autoimmune disease, because hyperactivation of B cells seems to play an important role in autoimmune diseases, irrelevantly whether it is the result of hyperfunction of B cells themselves or of hyperactivation of B cells by abnormal T cells. In the present study, administration of LhM in MRL/l mice reduced anti-DNA antibody production, but did not affect anti-Sm antibody production and most T cell abnormalities. These findings suggest that LhM cannot interfere with the development of autoimmune competent B cells but selectively suppress some B cell subsets for autoantibody production. However, activation of some suppressor T cell subsets may be also possible in this system. The mechanism of therapeutic effect on autoimmune diseases by LhM is further under investigation.

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和文抄録

金銭草の自己免疫病に対する作用を MRL/l をモデルマウスとして用いて検討した。金銭草の経口投与により（1500 mg/kg），脾とリンパ節の重量および脾細胞数は非処理群に比較して，有意な減少を示した。血清抗 ds-DNA 抗体については，金銭草投与群は非投与群と比較して有意な低下を示したが，抗 Sm 抗体については，有意差を示さなかった。すなわち，金銭草の効果は選択的な B 細胞 subsets の機能抑制，および抑制T細胞の誘導促進を介してであることが示された。なお，金銭草投与群においては非投与群に比して IL-2 産生能の有意の増強が認められたが，この効果が自己免疫病の発症抑制にどのような関わりを持つかについては検討中である。

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