The effects of Kampo formulae on the differentiation of intrathymic T lymphocytes in autoimmune mice

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Abstract

We focused on the important and basic 6 Kampo formulae, Mao-to (MOT ; 麻黄湯), Shimbu-to (SBT ; 真武湯), Ninjin-to (NJT ; 人参湯), Shigyaku-san (SGS ; 四逆散), Keishi-to (KST ; 桂枝湯), and Shimotsu-to (SMT ; 四物湯) and compared their pharmacological activity to examine the specificity of actions of Kampo formulae. In this study, we investigated the effects of these formulae on the immune system in ovariectomized mice and MRL/Mp-*lpr*/*lpr* (MRL) mice.

The alterations of intrathymic T cell subpopulations were observed in ovariectomized mice, where $CD4^+CD8^+$ cells decreased and the other subpopulations ($CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$) increased. These alterations were inhibited by NJT-or SGS-treatment. MOT tended to accelerate the alteration. Quite similarly, a decrease in $CD4^+CD8^+$ and increases in the other subpopulations were also observed in the thymus of MRL mice. Further, increases and decreases in $CD4^+CD8^+$ and $CD4^-CD8^+$, respectively, were observed in NJT- and SGS-treated groups. Splenic $\gamma\delta$-T lymphocyte tended to be lower in PSL-, SGS- and SMT- treated groups, however, these parameters are not always parallel with the results in thymocyte subpopulation.

Any decreases in circulating immune complexes (CICs) were not observed in all experimental groups. In addition, the difference was not statistically significant, however, CICs in the SGS-treated group were slightly higher than in the control.

Present data suggests that NJT and SGS improve the abnormalities in intrathymic T lymphocytes in the models of the climacteric disorder and the autoimmune diseases.

Key words Kampo formulae, Autoimmune disease, ovariectomy, thymus, T lymphocytes, MRL/lpr mice.

Abbreviations B6, C57BL/6 ; CICs, Circulating immune complexes ; KST, Keishi-to, 桂枝湯 ; MOT, Mao-to, 麻黄湯 ; MRL, MRL/Mp-*lpr*/*lpr* ; NJT, Ninjin-to, 人参湯 ; OVX, ovariectomy ; PBS, phosphate buffered saline ; SBT, Shimbu-to, 真武湯 ; SGS, Shigyaku-san, 四逆散 ; SLE, systemic lupus erytematosus ; SMT, Shimotsu-to, 四物湯.

Introduction

Recently, it has been reported that various Kampo-medicines were effective for prevention and therapy of several diseases and that the mechanisms of their actions were revealed by basic studies. However, these studies did not aim to clarify the specificity of each Kampo formula but the actions of Kampo formulae which have been clinically used for a long time. There are numerous combinations of herbs in Kampo-medicine. However their formulae can be categorized into a few basic formulae from a genealogical point of view. We focused on the important and basic 6 Kampo formulae which differ in their utility and contain relatively less kinds of herbs. Mao-

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to (麻黄湯), Shimbu-to (真武湯), Ninjin-to (人参湯), Shigyaku-san (四逆散), Keishi-to (桂枝湯), and Shimotsu-to (四物湯) are not often used clinically by themselves, however, these formulae are the basis of many widely used Kampo formulae. The objective of this investigation is to examine the specificity of actions of Kampo formulae by comparing the pharmacological activity of these 6 formulae.

Many autoimmune diseases in women have two peaks of incidence during periods of major hormonal changes, first after puberty and the second one at menopause.[1-3] It is clear that sex steroids regulates the immune system, since the immune response is altered by pregnancy, gonatectomy and hormone therapy[3-5] and the onset of certain autoimmune diseases are inhibited by estorogen administration.[6-8] Ovariectomy is useful model for the climacteric disorder. The alterations in the immune system observed in this model may be mediated with the pathogenesis of autoimmune disease. On the other hand, MRL/Mp-*lpr/lpr* (MRL) mice are models for human systemic lupus erytematosus (SLE). This strain spontaneously develops the autoimmune disease with massive lymphadenopathy characterized by immune-complex glomerulonephritis, arthritis, vasculitis, hypergammagloburinemia and the production of autoantibodies to nucleic acid.[9-12]

In this study, we investigated the effects of the basic 6 Kampo formulae on the immune system in ovariectomized mice and MRL mice.

Materials and Methods

Animals : C57BL/6N Jcl, C3H/He Jcl and Jcl : ICR female mice were obtained from Clea Japan, Inc. at 7 weeks of age. MRL/Mp-*lpr/lpr* female mice were obtained from Charles River Japan Inc. at 8 weeks of age. The animals were kept in plastic cages with wood shavings, 4-6 each, maintained in an animal room, which was air-conditioned (24-25°C) and artificially illuminated (12 hours of light from 8 : 00 AM to 8 : 00 PM) and provided with standard commercial pellet and tap water *ad libitum*.

Treatments : At 8 weeks of age, C57BL/6 (B6) mice were ovariectomized or sham-operated after i.p. injection of 50 mg/kg of sodium pentobarbital (Dainippon, Osaka, Japan). The sham-operated and ovariectomized control B6 mice were provided tap water *ad libitum* throughout the experiment. The experimental mice were given extracts (kindly provided by Tsumura, Co.) of Mao-to (MOT, TJ-27, lot #240027010 ; Glycyrrhizae Radix (1.5), Armeniacae Semen (5), Cinnamoni Cortex (4) and Ephedrae Herba (5)), Shimbu-to (SBT, TJ-30, lot #250020010 ; Paeoniae Radix (3), Zingiberis Rhizoma (1.5), Atractylodis Lanceae Rhizoma (3), Hoelen (4) and Aconiti Tuber (0.5)), Ninjin-to (NJT, TJ-32, lot #920032001 PO ; Zingiberis Siccatum Rhizoma (3), Glycyrrhizae Radix (3), Atractylodis Lanceae Rhizoma (3) and Ginseng Radix (3)), Shigyaku-san (SGS, TJ-35, lot #250035010 ; Glycyrrhizae Radix (1.5), Aurantii Fructus Immaturus (2), Bupleuri Radix (5) and Paeoniae Radix (4)), Keishi-to (KST, TJ-45, lot #230045010 ; Glycyrrhizae Radix (2), Cinnamoni Cortex (4), Paeoniae Radix Zingiberis Rhizoma (1.5) and Zizyphi Fructus (4)) and Shimotsu-to (SMT, TJ-71, lot # 250071010 ; Rehmanniae Radix (3), Paeoniae Radix (3), Cnidii Rhizoma (3) and Angelicae Radix (3)) at the concentration of 0.8 % as drinking water from 7 days before the ovariectomy. Three days after ovariectomy, mice were killed by decapitation under light ether anesthesia. MRL/Mp-*lpr/lpr* (MRL) mice were given samples from 9 weeks of age for 20 days as described above. Positive control mice were injected with prednisolone (2 mg/kg ; Sigma) subcutaneously 3 times every 3 day. Intact B6, C3H/He, ICR and MRL mice were also used for the flow cytemetric analysis 9, 8, 8 and 12 weeks of age, respectively.

Preparation of lymphocytes : At autopsy, the thymus and spleen was immediately removed, weighed and pressed with slide glass in phosphate buffered saline (PBS) (-). The cell suspension was passed through a #200 metal sieve and layered onto Ficoll-Conray solution (Immuno-Biological Laboratories, Fujioka, Japan) and centrifuged at 400×g for 30 min at room temperature. The interface layer was collected and washed 3 times with PBS(-).

Antibodies : Monoclonal antibodies used for flow cytometric analysis were R-phycoerythrin (PE) -conjugated rat anti-mouse CD4 IgG and fluorescein isothiocyanate (FITC) -conjugated rat anti mouse CD8a IgG, FITC-conjugated hamster anti-mouse $\alpha\beta$TCR

IgG, PE-conjugated rat anti-mouse γδTCR (Phamingen, San Diego, CA, USA).

Flow cytometric analysis : Splenic and thymic lymphocytes were incubated with 1 μg/million cells of fluorescein-conjugated antibodies for 1 h at 4°C. Fluorescence-activated cells were washed 3 times with PBS(-) and analyzed by an EPICS Elite flow cytometer (Coulter Cytometry Co., Hialeah, FL, USA). A fluorescence histogram of at least 5,000 counts was collected in each sample.

Circulating immune complexes : Before and after the treatment of samples, blood was collected from the retro-orbital plexus. Each blood sample was assayed for CICs by anti-C3b ELISA method.[13]

Statistics : The difference in each parameter among the groups was evaluated by Student's *t*-test or analysis of variance.

Results

Alterations of the thymocyte subpopulations in ovariectomized mice (Fig. 1)

Thymocyte subpopulations were determined on day 1, 3, 5 and 7 after the ovariectomy (OVX). $CD4^+CD8^+$ cells decreased with a peak at 3 days after OVX. On the other hand, $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were increased at 3 days after OVX. In addition, little differences were seen in the

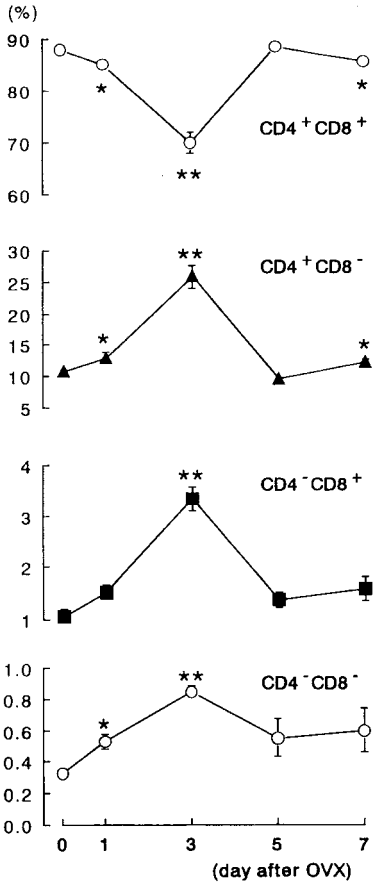

Fig. 1 Time course study of the intrathymic T cell subpopulations in ovariectomized mice (mean ± S.E.). *or**Significantly different from the intact group (day 0) at $p<0.05$ or 0.01.

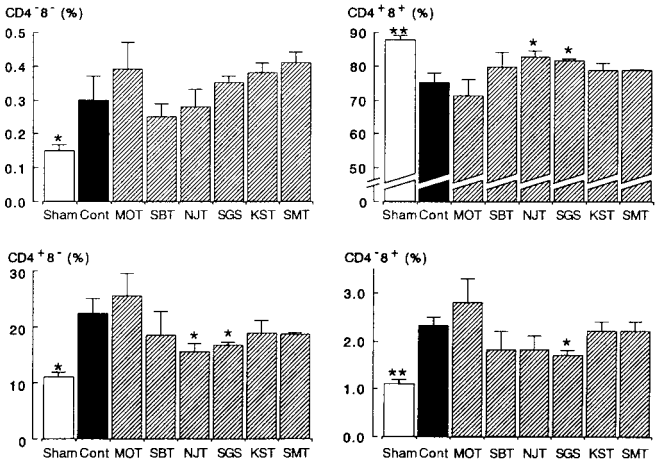

Fig. 2 The effects of Kampo formulae on the intrathymic T cell subpopulations in ovariectomized mice (mean±S.E.). *or**Significantly different from the ovariectomized control (Cont) at $p<0.05$ or 0.01. Sham : Sham-operated, Cont : Ovariectomized control, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

Table I Thymocyte subpopulation in MRL and other normal mice (Mean±S.E.)

Strain	Number of mice	$CD4^-8^-$	$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$
C57BL/6	4	0.32 ± 0.02^a	87.9 ± 0.5^a	10.7 ± 0.4^a	1.1 ± 0.1^a
C3H/He	4	0.65 ± 0.10^a	88.0 ± 1.8^a	9.1 ± 1.5^a	2.4 ± 0.3^a
ICR	5	2.68 ± 0.52^a	79.8 ± 2.1^a	14.9 ± 1.7^a	2.6 ± 0.1^a
MRL/Mp	6	7.58 ± 1.37^b	51.2 ± 2.7^b	23.6 ± 2.0^b	17.7 ± 1.2^b

^{a,b}Values with different superscripts are significantly different at $p<0.05$.

thymocyte subpopulations between intact and sham-operated mice (data not shown).

Body weight, water intake and dose in ovariectomized B6 mice

In all groups, there were no significant differences in body weight, while water intake was apparently lower in the MOT-treated group (data not shown). Doses (g/kg/day) of Kampo medicines calculated from water intakes were MOT : 1.4, SBT : 1.5, NJT : 1.9, SGS : 1.9, KST : 2.0 and SMT : 2.0.

Effects of the Kampo medicines on the thymocyte subpopulations in ovariectomized mice (Fig. 2)

Ovariectomized control mice showed significantly higher $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ and lower $CD4^+CD8^+$ than sham-operated mice. While, in the NJT-and SGS-treated mice, significant increases of $CD4^+CD8^+$ and decreases of $CD4^+CD8^-$ and $CD4^-CD8^+$ were observed, compared to the control. These indicate that NJT and SGS inhibited the alteration of thymocyte subpopulation induced by OVX. In the MOT - treated group, $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ tended to increase, compared to the control, however, the differences were not statistically significant.

Thymocyte subpopulation in MRL mice (Table I)

To examine if the alterations in thymocyte subpopulation observed in ovariectomized mice are involved in the pathogenesis of autoimmune diseases, the thymocyte subpopulation in MRL mice, a model for human systemic lupus erythematosus, were compared with mice regarded as immunologically normal. Similarly to ovariectomized mice, MRL mice showed significantly higher $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ and lower $CD4^+CD8^+$ than B6, C3H/He and ICR.

Body weight, water intake and dose in MRL mice (Table II)

Table II Effects of Kampo formulae on body weight and water intake in MRL mice

Group	Body Weight (mean ±S.E.)		Water intake (mean) (g/mouse/day)
	Initial (g)	Final (g)	
Control	26.8±0.8	29.3±1.1	6.42
PSL	27.0±0.7	28.2±0.7	7.01
MOT	26.8±0.7	24.7±0.8**	2.57
SBT	27.3±0.6	29.3±0.8	4.40
NJT	27.3±0.8	29.5±1.2	5.07
SGS	27.0±0.9	29.4±1.7	5.20
KST	26.9±0.9	30.2±0.6	4.99
SMT	26.9±1.0	29.0±1.4	6.60

n=6 in each group. **Significantly different from the control at $p<0.01$. PSL : prednisolone, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT: Shimotsu-to, 四物湯.

Table III Effects of Kampo formulae on weights of thymus and spleen in MRL mice (Mean±S.E.)

Group	Thymus (mg)	Spleen (mg)
Control	64.2±2.50	239±18.1
PSL	34.0±1.91**	246±39.8
MOT	50.9±3.32**	190±16.6
SBT	56.4±1.80*	264±55.0
NJT	57.0±2.02*	287±25.4
SGS	53.2±2.71**	428±98.9*
KST	57.7±1.97*	229±11.9
SMT	54.1±3.93*	276±21.7

n=6 in each group. *or **Significantly different from the control at $p<0.05$ or 0.01. PSL : prednisolone, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

In the MOT-treated mice, body weight and water intake were apparently lower than in the control group. Doses (g/kg/day) of Kampo medicines calculated from water intakes were MOT : 0.7, SBT : 1.2, NJT : 1.4, SGS : 1.4, KST : 1.4 and SMT : 1.9.

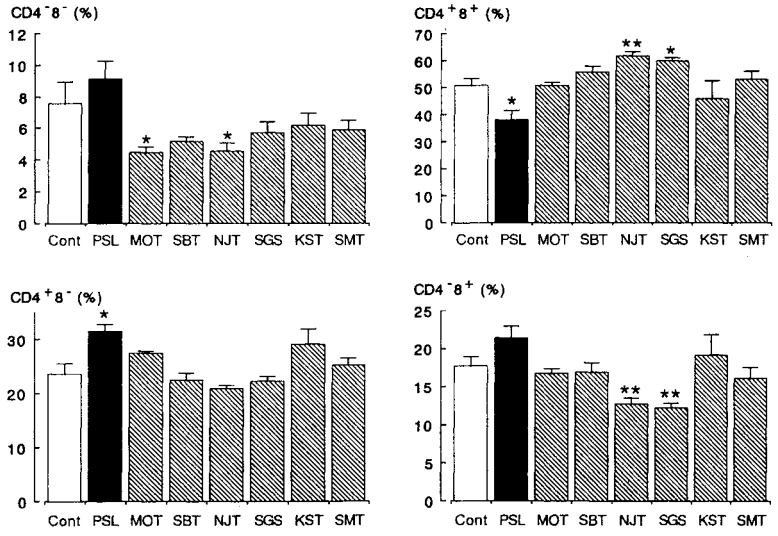

Fig. 3 The effects of Kampo formulae on the intrathymic T cell subpopulation in MRL mice (mean±S.E.). *or**Significantly different from the control at $p<0.05$ or 0.01. (Cont : Control, PSL : prednisolone, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

Weights of thymus and spleen (Table III)

Thymus weights were significantly lower in PSL-treated and all experimental groups, where these differences were marked in PSL-, MOT- and SGS-treated groups. In spleen weights, the SGS-treated group was significantly higher than the control.

Effects of Kampo medicines on thymocyte subpopulation in MRL mice (Fig. 3)

In the NJT-and SGS-treated mice, $CD4^+CD8^+$ and $CD4^-CD8^+$ cells were significantly increased and decreased, respectively, compared to the control. $CD4^-CD8^-$ cells were significantly lower in the MOT- and NJT-treated group than in the control. In the PSL-treated group, $CD4^+CD8^+$ and $CD4^+CD8^-$ were further decreased and increased, respectively.

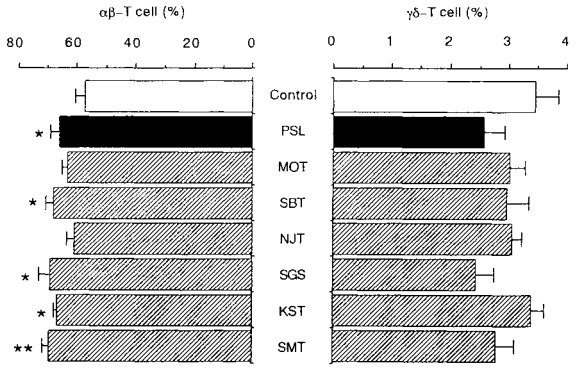

Fig. 4 The effects of Kampo formulae on the $\alpha\beta$-and $\gamma\delta$-T lymphocytes in MRL mice (mean±S.E.). *or**Signifi cantly different from the control at $p<0.05$ or 0.01. Cont : Control, PSL : prednisolone, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

$\alpha\beta$- and $\gamma\delta$-T lymphocytes (Fig. 4)

$\alpha\beta$-T lymphocytes in the spleen were significantly higher in the PSL-, SBT-, SGS-, KST- and SMT-treated group than in the control. In the PSL-, SGS- and SMT-treated group, $\gamma\delta$-T lymphocytes tended to

Table IV Effects of Kampo fomulae on CICs (Mean±S.E.)

Group	CIC (O.D. 405nm)		Change (%)
	Initial	Final	
Control	0.160±0.061	0.156±0.044	121±21
PSL	0.140±0.024	0.151±0.034	110±19
MOT	0.135±0.024	0.140±0.022	125±29
SBT	0.109±0.024	0.140±0.022	130±18
NJT	0.123±0.011	0.130±0.015	115±8
SGS	0.155±0.069	0.223±0.041	190±57
KST	0.093±0.007	0.137±0.023	155±33
SMT	0.095±0.013	0.147±0.022	159±14

n=6 in each group. PSL : prednisolone, MOT : Mao-to, 麻黄湯, SBT : Shimgu-to 真武湯, NJT : Ninjin-to, 人参湯, SGS : Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

decrease compared to the control, however, these differences were not statistically significant.

Circulating immune complexes (CICs ; Table IV)

There were not any significant differences in CICs between the control and experimental groups, however, CICs in the SGS-treated group were slightly higher than in the control.

Discussion

Inactivation of intrathymic T cell differentiation was observed by aging,[14] bacterial infection,[15] estrogen administration[16,18] and malignancies.[19,21] These data showed that $CD4^+CD8^+$ cells and the other subpopulation in the thymus were decreased and increased, respectively.[17,21] Extrathymic T lymphocytes often develop at the thymic involution phase[14,17,20] and, in some cases, contain $\gamma\delta$-[14] or self-reactive[15,17] cells, which in turn are responsible for certain autoimmune diseases.

In this study, the alterations of intrathymic T cell subpopulations were observed in ovariectomized mice, where $CD4^+CD8^+$ cells decreased and the other subpopulations increased, similarly to the published data described above.[17,21] Imbalance in sex steroids in ovariectomized mice was thought to induce these changes, through the specific receptors for gonadal steroids in thymus.[22-24] These alterations were improved by the NJT- or SGS-treatment. Thus, this data suggests that NJT and SGS inhibit the abnormal intratymic T cell differentiation at the climacteric phase and possibly prevents the onset of autoimmune diseases.

It is known that pathogenesis of diseases in MRL mice is thymus-dependent.[25] Neonatal thymectomy prevents lymphadenopathy, nephritis and production of autoantibodies to DNA and immune-complexes and improves survival rate in this strain.[26] Quite similarly to the ovariectomized mice, a decrease in $CD4^+CD8^+$ and increases in the other subpopulations were also observed in the thymus of MRL mice. Further, increases and decreases in $CD4^+CD8^+$ and $CD4^-CD8^+$, respectively, were observed in the NJT- and SGS-treated group. Thus, our data suggests that NJT and SGS improve the defects of intrathymic differentiation in MRL mice. In addition, PSL seems to increase the abnormality in thymocyte subpopulation in this model.

A significant fraction of $\gamma\delta$-T lymphocyte is regarded to differentiate extrathymicly and recognize self-reactive antigens in mice.[27] It is also reported that $\gamma\delta$-T lymphocytes increase in patients with some autoimmune diseases such as Sjogren's syndrome,[28] Hashimoto's thyroiditis,[29] multiple sclerosis[30] and celiac disease.[31] Present data showed that $\gamma\delta$-T lymphocyte tended to be lower in the PSL-, SGS- and SMT-treated group, however, these parameters are not always parallel with the thymocyte subpopulation.

Immune complexes are one of the pathogenesis of autoimmune disease. Circulating immune complexes (CICs) are frequently detected in patients with autoimmune diseases such as systemic lupus erythematosus,[32,33] rheumatoid arthritis[34] and others. The deposition of immune complexes in the tissues led to glomerulonephritis, vasculitis and skin diseases.[35,36] In this study, any decreases in CICs were not observed in all experimental groups. This was perhaps due to the age of the mice, 9 weeks of age at the beginning of the experiment, in which the disease of the mice might have already progressed. In addition, the difference was not statistically significant, however, CICs in the SGS-treated group was slightly higher than in the control. Since an increase in spleen weight was also observed in SGS-treated group, we cannot deny the possibility that the SGS accelerates the disease by its immunopotentiation.

Taken together, NJT may regulate or normalize the abnormal intrathymic differentiation and their actions are clearly different from PSL.

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和文抄録

我々は漢方方剤の作用特異性を明らかにすることを目

的として，6種の漢方方剤（麻黄湯，真武湯，人参湯，四逆散，桂枝湯および四物湯）を選び出し，薬理作用を比較検討している。本研究では，卵巣摘出およびMRL/Mp-*lpr*/*lpr*（MRL）マウスの免疫系に対するこれら6方剤の影響を検討した。

卵巣摘出マウスにおいては胸腺内T細胞サブポピュレーションに変化がみられ，$CD4^+CD8^+$が低下し，その他のサブポピュレーション（$CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$）はいずれも増加した。これらの変化は人参湯および四逆散投与により抑制された。反対に，麻黄湯はこれらの変化を促進する傾向がみられた。同様に，MRLマウスの胸腺においても$CD4^+CD8^+$の低下およびその他のサブポピュレーションの増加が観察されたが，人参湯および四逆散投与により，$CD4^+CD8^+$の増加および$CD4^-CD8^+$の減少が認められた。脾臓$\gamma\delta$-Tリンパ球はプレドニゾロン，四逆散および四物湯投与により低下する傾向がみられたが，胸腺内の結果とは必ずしも一致しなかった。

血中免疫複合体量はいずれの群においても有意な減少は認められなかったが，四逆散投与群においては増加傾向がみられた。

これらの結果は人参湯および四逆散が更年期障害および自己免疫疾患モデルマウスの免疫系，特に胸腺内T細胞の異常を改善する可能性を示唆している。

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