Studies of Kampo medicines on the clearance of immune complexes in experimental autoimmune mice

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Abstract

In order to investigate the therapeutic effects of Kampo medicines, effects of Kampo medicines on the clearance of immune complexes (ICs) in experimental autoimmune mice were examined.

GVHR-induced mice, which were one of the models of autoimmune disease increased the value of circulating immune complexes (CICs). However, increments of CICs observed in GVHR-induced mice were decreased on Toki-shakuyaku-san (当帰芍薬散 ; TSS)-, Kihi-to (帰脾湯 ; KHT)- and Keishi-ninjin-to (桂枝人参湯 ; KNT)-treated mice. In histological studies, proliferation of lymphocytes observed in the spleen of GVHR-induced mice decreased in TSS-, KHT- and KNT- treated mice. Further, increments of spleen weight observed in GVHR-induced mice were decreased in TSS-, KHT-, KNT- and Kami-shimotsu-to (加味四物湯 ; KST)-treated mice. In flow cytometric analysis, TSS prevented infiltration and/or proliferation of donor lymphocytes in the spleen of recipients.

Using animal model of lupus, MRL Mp-lpr/lpr mice, effects of Kampo medicines on the clearance of ICs were examined *in vivo*. The clearance of TSS-treated MRL Mp-lpr/lpr mice was significantly accelerated, in comparison with control mice. In time course studies on the changes of CICs, TSS-treated mice tended to decrease CICs.

In order to investigate the active component(s) of TSS, effects of TSS and its component herbs on the binding of ICs to macrophages were demonstrated *in vitro*. The binding of ICs to macrophages was significantly enhanced by TSS in dose-dependent manner. On the other hand, "TSS minus Angelicae Radix" decoction represented the significant decrease of the binding of ICs compared to TSS treatment. Furthermore, the enhancing activity was only observed in the combination of Angelicae Radix and Atractylodis Lanceae Rhizoma.

These results indicate that the clinical effects of Kampo medicines against autoimmune diseases may result in partly from increment of the clearance of ICs from the circulation.

Key words autoimmune diseases, immune complexes, experimental models.

Introduction

Circulating immune complexes (CICs) are commonly detected in the patients with autoimmune diseases *i.e.* systemic lupus erythematosus (SLE)[1,2] rheumatoid arthritis[3] and the others. The deposition of immune complexes (ICs) to tissues has been considered to be one of the pathogenesis of autoimmune diseases, and to be a causative agents of glomerulonephritis, vasculitis and skin diseases.[4,5] These facts suggest that increment of the clearance of ICs from the circulation have improved pathogenesis of autoimmune diseases.

In general, steroids have been used for treatment of autoimmune diseases. However, such drugs have severe adverse reactions and rebound phenomena. Thus, considerable efforts to find new drugs which improve pathogenesis of autoimmune diseases have been engaging in a number of researchers. Previously,

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we reported[6] that steroid-treated mice decreased the clearance of ICs. When Sairei-to (柴苓湯 ; SRT) were administered to the steroid-treated mice, the clearance of ICs was increased in SRT-treated mice.

In addition, some clinical reports[7] have indicated that the traditional Japanese (Kampo) medicines improved the pathogenesis in the patients with autoimmune diseases. However, the mode of action of Kampo medicines have been still unclear.

It has been hypothesized that one of the clinical effects with Kampo medicines may been associated with acceleration of the clearance of ICs from the circulation. In order to examine the mechanisms of action of Kampo medicines, effects of Kampo medicines on the clearance of ICs in experimental autoimmune mice were investigated. In this studies, GVHR-induced autoimmune-like mice and MRL Mp-lpr/lpr mice were used in these experiments. Further, to investigate the active component(s) in Kampo medicines, the binding of ICs to macrophages were demonstrated *in vitro*.

I. Effects of Kampo medicines on graft *versus* host reaction (GVHR) mice[8]

GVHR mice indicate human SLE-like pathogenesis including persistent lymphoid hyperplasia, hypergammaglobulinemia, production of autoantibodies and glomerulonephritis. Therefore, it was considered that GVHR-induced mice were one of the models of autoimmune disease. In this studies, effects of Kampo medicines against GVHR-induced mice were investigated.

GVHR was induced according to the procedure of Rolink with a slight modification.[9] Namely, (C57BL/6×DBA/2) F_1 (BDF_1) mice were injected parental splenocytes for 3 times at 6 days intervals and then GVHR was induced. Six kind of Kampo medicines, which were Toki-shakuyaku-san (当帰芍薬散 ; TSS), Shigyaku-san (四逆散 ; SGS), Jyuzen-taiho-to (十全大補湯 ; JTT), Kihi-to (帰脾湯 ; KHT), Keishi-ninjin-to (桂枝人参湯 ; KNT) and Kami-shimotsu-to (加味四物湯 ; KST) were orally administered with each groups of GVHR-induced mice. Experimental design is shown in Fig. 1.

No significant differences between control and Kampo medicines treated groups was observed in urine protein level (data not shown). However, increments of CICs observed in GVHR-induced mice was decreased on TSS-, KHT-and KNT-treated groups (Fig. 2).

In histological studies, proliferation of lymphocytes was observed in the spleen of GVHR-induced mice. However, these proliferation observed in the spleen decreased in TSS-, KHT- and KNT-treated groups (data not shown). Further, increment of spleen

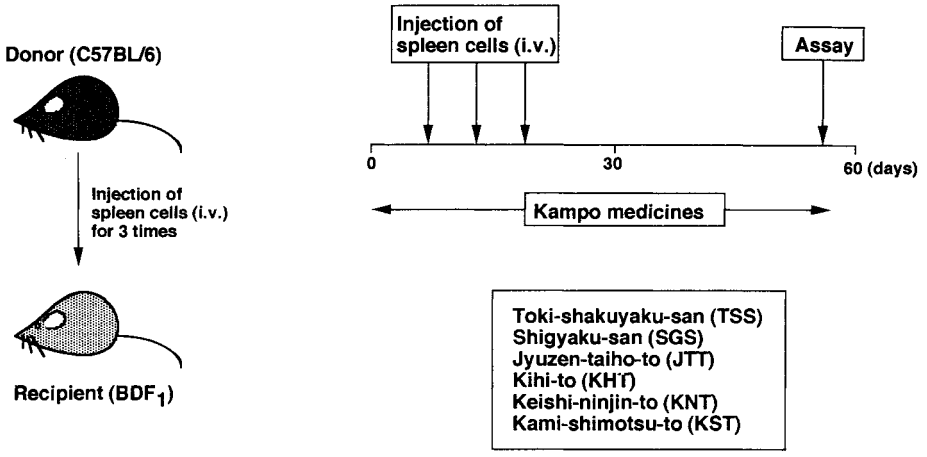

Fig. 1 Experimental design on investigation of effects of Kampo medicines against acute GVHR-induced mice.

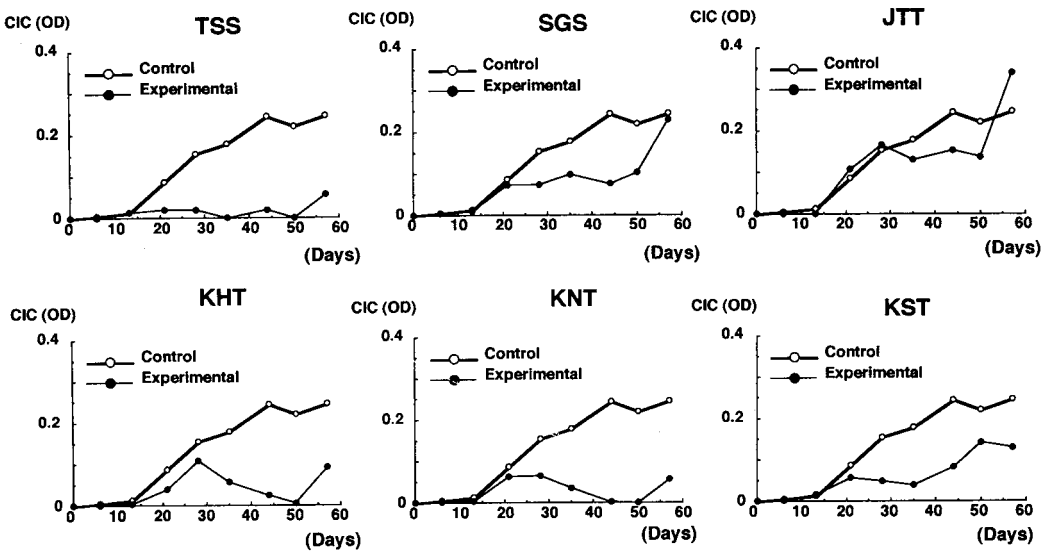

Fig. 2 Effects of Kampo medicines on the change of circulating immune complexes.
TSS ; Toki-shakuyaku-san, SGS ; Shigyaku-san, JTT ; Jyuzen-taiho-to,
KHT ; Kihi-to, KNT ; Keishi-ninjin-to, KST ; Kami-shimotsu-to

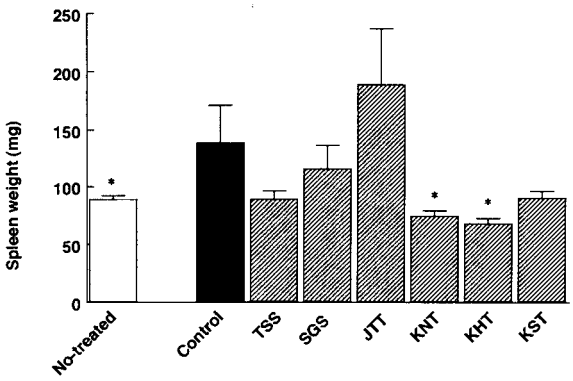

Fig. 3 Effects of Kampo medicines on increment of spleen weight.
TSS ; Toki-shakuyaku-san, SGS ; Shigyaku-san, JTT ; Jyuzen-taiho-to, KHT ; Kihi-to, KNT ; Keishi-ninjin-to, KST ; Kami-shimotsu-to
Each value expresses mean±S.E. (n=10)
Significant difference from the control $*p<0.05$

weight observed in GVHR-induced mice was significantly decreased in KHT-and KNT-treated groups, and TSS-and KST-treated groups also tended to decrease spleen weight (Fig. 3).

Next, infiltration of transplanted lymphocytes in the spleen of GVHR-induced mice (recipient mice) were measured in TSS-treated group. Lymphocytes derived from donor mice (C57BL/6) have expressed $H2K^b$ MHC antigen and lymphocytes derived from recipient mice (BDF_1) have expressed $H2K^{bd}$ MHC antigen, respectively. Therefore, distinguishing lymphocytes between recipient and donor have been determined in flow cytometric analysis. The results are shown in Fig. 4. Lymphocytes obtained from no-treated mice were expressed $H2K^{bd}$ MHC antigen only, however lymphocytes obtained from GVHR-induced mice (control) were expressed either $H2K^b$ MHC antigen and $H2K^{bd}$ MHC antigen ($H2K^b$ MHC ; 88.6 %, $H2K^{bd}$ MHC ; 8.4 %). On the other hand, the expression of $H2K^b$ MHC antigen decreased in lymphocytes obtained from TSS-treated mice compared to control ($H2K^b$ MHC ; 23.1 %, $H2K^{bd}$ MHC ; 76.7 %).

These results suggested that TSS, KHT and KNT prevented infiltration and/or proliferation of donor lymphocytes in the spleen of recipients. Furthermore, it was suggested from above-described that these Kampo medicines accelerated the clearance of ICs from the circulation. The clinical effects of Kampo medicines against autoimmune diseases may result in partly from these action of Kampo medicines.

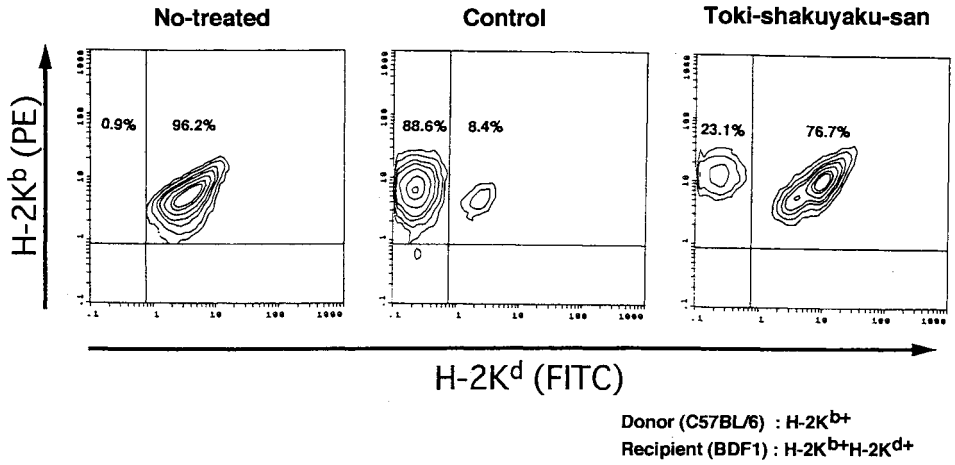

Fig. 4 Effects of Toki-shakuyaku-san on the infiltration of donor lymphocytes

Mice were injected with immune complexes (glucose oxidase anti-glucose oxidase complexes ; GAG) (i.v.)

↓

Plasma samples were obtained from retro-orbital plexus for 4 times (6, 10, 14, 18 min.)

↓

Plasma samples were added with substrate of glucose oxidase

↓

The activities of glucose oxidase were measured in plasma samples

↓

Half-life of GAG from the circulation (T1/2) were calculated

Fig. 5 Experimental design on investigation of the clearance of immune complexes *in vivo*

Table I Effect of Kampo medicines on the clearance of immune complexes *in vivo*

	T 1/2 (min.)
Control	13.0±1.50
Toki-shakuyaku-san	8.51±1.83*
Chorei-to	10.6±1.88
Keishi-bukuryo-gan	12.1±2.20
Sairei-to	12.6±1.94

Each value expresses mean±S.D. (n=5-7)
Significant difference from the control *$p<0.01$

II. Effects of Kampo medicines on MRL Mp-lpr/lpr mice[10]

In order to investigate the therapeutic effects of Kampo medicines, which were Toki-shakuyaku-san (TSS), Chorei-to (猪苓湯 ; CRT), Keishi-bukuryo-gan (桂枝茯苓丸 ; KBG) and Sairei-to (柴苓湯 ; SRT) against autoimmune diseases, the clearance of ICs was examined *in vivo*,[11] using animal model of lupus, MRL Mp-lpr/lpr mice.

Experimental design is shown in Fig. 5. Female MRL Mp-lpr/lpr mice had administered Kampo medicines at 10 weeks of age through their drinking water for 6 weeks (42 days). After 6 weeks, the clearance of ICs was examined. The results are shown in Table I. The value of clearance indicates half life (T 1/2) of ICs from the circulation. The clearance of TSS-treated group was significantly accelerated ($p<0.01$), however no significant differences were found in the other treated groups compared to control. The observations suggested that TSS should be a potential formulation on the clearance of ICs from the circulation.

Time course studies on the changes of anti-dsDNA antibodies and CICs were measured by ELISA. The result of time course study are shown in Table II. The value of TSS-treated group tended to decrease CICs. On the other hand, increment of anti-

Table II Time course study on the changes of circulation immune complexes

	0 day	7 day	14 day	21 day	28 day	38 day
Control	0.222±0.055	0.257±0.068	0.290±0.055	0.308±0.080	0.290±0.091	0.273±0.074
TSS	0.229±0.088	0.217±0.103	0.235±0.090	0.249±0.095	0.233±0.057	0.194±0.055[a)]

TSS ; Toki-shakuyaku-san All data indicated optical density at 405 nm. Values are the mean±S.D.(n=6~7)
[a)] Statistical differences between the mean of treated (TSS) and control. P value is 0.078.

dsDNA antibodies have not improved with TSS treatment (data not shown).

These results suggested that TSS may improve the physical condition of patients with autoimmune diseases through enhancing the clearance of ICs from the circulation, but without suppressing the production of auto-antibodies.

III. Effects of combination on the component herbs of TSS[12, 13)]

Mice were injected with thioglycollate medium (i.p.)
↓ 96 hr
Macrophages were obtained from peritoneal cavity
↓
Macrophages were suspended with RPMI 1640 medium
↓ Cultured in 96 well microplate
Added with samples
↓ 37°C, 5 % CO_2 20 hr incubate
Added with immune complexes (peroxidase anti-peroxidase complexes ; PAP)
↓ 4°C, 4 hr incubate
↓ Washed with PBS for 3 times
↓ Added with Nonidet P-40
↓ Added with substrate of peroxidase
The binding of PAP to macrophages were measured in the optical density (O.D. 492 nm)

Fig. 6 Protocol design on investigation of the binding of immune complexes to macrophages *in vitro*

It is considered that ICs are mainly removed by reticuloendotherial cells including macrophages *via* binding to Fc receptors and/or C3 receptors. Next, in order to investigate the active component(s) of TSS, effects of TSS and component herbs on the binding of ICs to macrophages were demonstrated *in vitro*.[14)] Experimental protocol is shown in Fig. 6. The binding of ICs to macrophages was significantly enhanced by TSS in dose-dependent manner (Fig. 7). In the studies of "TSS minus one crude drug", "TSS minus Angelicae Radix" decoction represented the significant decrease of the binding of ICs compared to TSS treatment (Table III). Furthermore, the results of the *in vitro* experiment accord with that of the *in vivo* study[13)] (Table IV). These results indicate that Angelicae Radix significantly contributes to increments of the clearance of ICs with TSS. Thus, effects of combination of two crude drugs was then assayed. As

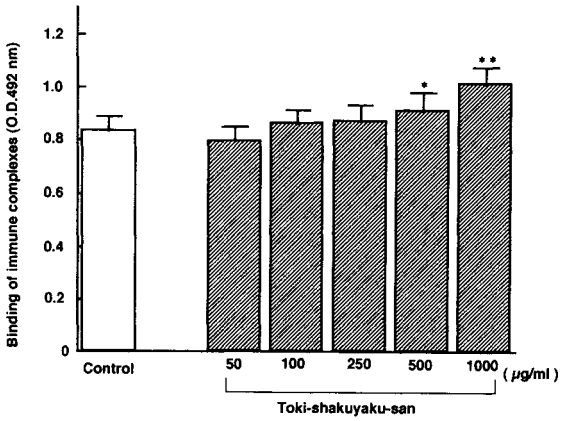

Fig. 7 Effects of Toki-shakuyaku-san on the binding of immune complexes to macrophages *in vitro*
Each value expresses mean±S.D.(n=8)
Significant difference from the control $^{*}p<0.05$, $^{**}p<0.01$

Table III Effect of Toki-shakuyaku-san excluding individual components on the binding of immune complexes to macrophages *in vitro*

	Immune complexes binding (O.D. 492 nm)
Control	0.712±0.084*
TSS	1.017±0.109
TSS-AR	0.691±0.127*
TSS-PR	1.024±0.080
TSS-CR	1.104±0.092
TSS-H	1.096±0.078
TSS-ALR	0.912±0.097
TSS-ALI	1.112±0.122

TSS ; Toki-shakuyaku-san, AR ; Angelicae Radix, PR ; Paeoniae Radix, CR ; Cinidii Rhizoma, H ; Hoelen, ALR ; Atractylodis Lanceae Rhizoma, ALI ; Alismatis Rhizoma
Each value expresses mean±S.D. (n=8)
Significant difference from TSS ; $*p<0.001$

Table IV Effects of Toki-shakuyaku-san excluding individual components on the clearance of immune complexes *in vivo*

	T 1/2 (min.)
Control	13.0±2.50**
TSS	8.15±1.83
TSS-AR	13.1±2.31**
TSS-PR	10.5±2.58
TSS-CR	10.1±2.58
TSS-H	10.7±2.10
TSS-ALR	12.5±2.46*
TSS-ALI	10.7±2.00

TSS ; Toki-shakuyaku-san, AR ; Angelicae Radix, PR ; Paeoniae Radix, CR ; Cinidii Rhizoma, H ; Hoelen, ALR ; Atractylodis Lanceae Rhizoma, ALI ; Alismatis Rhizoma
Each value expresses mean±S.D. (n=8)
Significant difference from TSS ; $*p<0.05$, $**p<0.01$

shown in Table V, the enhancing activity was only observed in the combination of Angelicae Radix and Atractylodis Lanceae Rhizoma, and this result indicates that the combination of Angelicae Radix and Atractylodis Lanceae Rhizoma was the main contribute to such action of TSS.

In conclusion, the results demonstrated here indicate the possibility of TSS may have a potential therapeutic effects on treating patients with autoimmune diseases.

Table V Effect of "two crude drugs extract" on the binding of immune complexes to macrophages *in vitro*

	Immune complexes binding (O.D. 492 nm)
Control	0.695±0.043
TSS	0.877±0.048*
AR	0.703±0.045
AR+PR	0.734±0.045
AR+CR	0.691±0.027
AR±H	0.685±0.019
AR+ALR	0.809±0.043*
AR+ALI	0.686±0.022

TSS ; Toki-shakuyaku-san, AR ; Angelicae Radix, PR ; Paeoniae Radix, CR ; Cinidii Rhizoma, H ; Hoelen, ALR ; Atractylodis Lanceae Rhizoma, ALI ; Alismatis Rhizoma
Each value expresses mean±S.D. (n=8)
Significant difference from the control ; $*p<0.001$

Conclusion

In the present study, several effects of Kampo medicines against experimental autoimmune mice were demonstrated. Particularly, Toki-shakuyaku-san (TSS) improved both the clearance of ICs with MRL Mp-lpr/lpr mice and increment of CICs observed in GVHR-induced mice. TSS further prevented infiltration and/or proliferation of donor lymphocytes in the spleen of GVHR-induced mice. TSS is one of Kampo medicines frequently applied to gynaecological disorders. According to Kampo classic textbooks, TSS has been shown to improve ovarin dysfunction, and has been empirically used as a remedy for amenorrhea, luteal phase dysfunction, and anovulatory syndrome. The present study indicates that TSS may also show a potential therapeutic effects on autoimmune disorders.

In conclusion, the clinical effects of Kampo medicines against autoimmune diseases may result in partly from increment of ICs clearance.

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和文抄録

自己免疫疾患に対して種々の漢方方剤が臨床的に治療効果を示すことが報告されているが，その作用メカニズムについては明らかとされていない。そこで自己免疫疾患に対する漢方方剤の影響を調査するため，自己免疫疾患モデル動物を用いて検討を行った。Graft *versus* host reaction (GVHR) で引き起こされるマウス自己免疫疾患モデルに対し，当帰芍薬散，帰脾湯および桂枝人参湯は血中免疫複合体量の増加抑制作用を示した。またこれらの方剤は，GVHR モデルで観察された脾臓でのリンパ球の増殖および脾臓重量の増加を抑制した。さらにフローサイトメトリーによる解析で，当帰芍薬散は移植されたドナー由来のリンパ球の脾臓への浸潤，あるいは増殖を抑制することが明らかとなった。次に遺伝的自己免疫疾患モデルである MRL Mp-lpr/lpr マウスの免疫複合体除去能に対する漢方方剤の影響について検討した結果，当帰芍薬散は免疫複合体除去能の増加作用を示した。また当帰芍薬散は血中免疫複合体量の増加を抑制した。さらにマクロファージの免疫複合体結合能に対する検討で，当帰芍薬散は免疫複合体結合能の増加作用を示し，またこの作用発現には当帰と蒼朮を同時に抽出することが重要であると考えられた。

以上の結果より，漢方方剤による自己免疫疾患に対する臨床での病態改善効果の一部は，免疫複合体除去能の増加によることが示唆された。

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