Effects of Toki-shakuyaku-san on the expression of Fc receptors and CR3 on macrophages in mice

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Abstract

The action mechanisms of Toki-shakuyaku-san (TSS, Dang-Gui-Shao-Yao-San ; 当帰芍薬散), one of the Kampo medicines, on the clearance of immune complexes and macrophage function were investigated.

In the *in vivo* study, oral administration of TSS enhanced the immune complexes clearance from the circulation in MRL Mp-lpr/lpr mice and C3H/He mice, but no effrect was observed in the carbon clearance assay. In the *in vitro* study, TSS increased the binding of immune complexes to macrophages or Kupffer cells, and the digestion of immune complexes by Kupffer cells. In the experiment of flow cytometric analysis, the expressions of FcγII/III receptors and complement receptor 3 (CR3) on macrophages were increased by the treatment with TSS. Similar results were obtained by immunofluorescence microscopical studies.

In addition, it was also reported that the appearance of the activity was due to the combination of Angelicae Radix and Atractylodis Lanceae Rhizoma, two of the six ingredients of TSS. Both outer and inner dialysate of the extract of Angelicae Radix and Atractylodis Lanceae Rhizoma potentiated the binding of immune complexes to macrophages. Low molecular fraction was further fractionated by using column chromatography, and the active components were concentrated in fraction 5-C (named LMW5-C).

In conclusion, one of the mechanisms of enhancement of immune complexes clearance was thought to be due to an increase in the immune complexes binding to macrophage through augment of FcγII/III receptors and CR3 expression. It was revealed that the active components were not only high molecular substances but low molecular ones.

Key words Toki-shakuyaku-san, immune complexes, macrophages, phagocytosis, Kupffer cells, Fc receptors, C3 receptors.

Abbreviations AAP, 4-aminoantipyrine ; ALR, Atractylodis Lanceae Rhizoma ; AR, Angelicae Radix ; CR3, complement receptor 3 ; FCS, fetal calf serum ; FITC, fluorescein isothiocyanate ; GAG, glucose oxidase anti-glucose oxidase complex ; HMW, high molecular weight fraction ; LMW ; low molecular weight fraction ; OPD, *o*-phenylenediamine dihydrochloride ; PAP, peroxidase anti-peroxidase ; PBS, phosphate buffered-saline ; TSS, Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San), 当帰芍薬散.

Introduction

Immune complexes are one of the pathogenesis of autoimmune diseases. The deposition of immune complexes in the tissues led to glomerulonephritis, vasculitis and skin diseases.[1,2] Circulating immune complexes are frequently detected in patients with autoimmune

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diseases such as systemic lupus erythematosus,[3,4] rheumatoid arthritis[5] and others. In our previous studies, we reported that Toki-shakuyaku-san (TSS, Dang-Gui-Shao-Yao-San ; 当帰芍薬散) and the combined extract of Angelicae Radix and Atractylodis Lanceae Rhizoma, which are two of the six ingredients of TSS, increased immune complexes binding to macrophages *in vitro*.[6] However, the mode of action of TSS on the clearance of immune complexes and the active components are still unclear.

It is considered that immune complexes are mainly removed by reticuloendothelial cells including Kupffer cells, spleen macrophages and the other tissue macrophages *via* binding to Fc receptors and/or C3 receptors. Therefore, in the present report, the effects of TSS on the binding of immune complexes to Kupffer cells and on immune complexes digestion by Kupffer cells were investigated. Further, the actions of TSS on immune complexes clearance (specific phagocytosis) and carbon clearance (non-specific phagocytosis) in non autoimmune diseases mice were studied, and the influence of TSS on the expression of Fc receptors and C3 receptors on macrophages were examined using flow cytometric analysis and immunofluorescence methods.

In addition, to examine the active components in TSS, we proceeded to fractionate the combined extract of Angelicae Radix and Atractylodis Lanceae Rhizoma by testing immune complexes binding assay.

Materials and Methods

Crude drugs : Crude drugs were purchased from Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan) and Tsumura Co. Ltd. (Tokyo Japan). The recipes of each formulation, Toki-shakuyaku-san (TSS), Chorei-

Table I Kampo formulations

	Species	Latin name	Weight (g)
TSS	*Alisma orientale* Juzepc.	Alismatis Rhizoma	4.0
	Angelica acutiloba Kitagawa	Angelicae Radix	3.0
	Atractylodes lancea DC.	Atractylodis Lanceae Rhizoma	4.0
	Cnidium officinale Makino	Cnidii Rhizoma	3.0
	Poria cocos Wolf.	Hoelen	4.0
	Paeonia latiflora Pall.	Paeoniae Radix	4.0
CRT	*Alisma orientale* Juzepc.	Alismatis Rhizoma	3.0
	Equus aninus L.	Asini Gelatinum	3.0
	Poria cocos Wolf.	Hoelen	3.0
	Hydrate halloysite	Kadinum	3.0
	Polyporus umbellatus Fries	Polyporus	3.0
KBG	*Cinnamomum cassia* Blume	Cinnamomi Cortex	4.0
	Poria cocos Wolf.	Hoelen	4.0
	Paeonia suffruticosa Andrews	Moutan Cortex	4.0
	Paeonia latiflora Pall.	Paeoniae Radix	4.0
	Prunus persica Batsch	Persicae Semen	4.0
SRT	*Alisma orientale* Juzepc.	Alismatis Rhizoma	4.0
	Atractylodes japonica Koidzumi	Atractylodis Rhizoma	2.5
	Bupleurum falcatum L.	Bupleuri Radix	5.0
	Cinnamomum cassia Blume	Cinnamomi Cortex	2.0
	Panax ginseng C.A. Meyer	Ginseng Radix	2.5
	Glycyrrhiza glabra L.	Glycyrrhizae Radix	2.0
	Poria cocos Wolf.	Hoelen	2.5
	Pinellia ternata Breitenbach	Pinelliae Tuber	4.0
	Polyporus umbellatus Fries	Polyporus	2.5
	Scutellaria baicalensis Georgi	Scutellariae Radix	2.5
	Zingiber officinale Roscoe	Zingiberis Rhizoma	0.5
	Zizyphus jujuba Mill. var. *inermis* Rehd	Zizyphi Fructus	2.5

to (CRT, Zhu-Ling-Tang ; 猪苓湯), Keishi-bukuryo-gan (KBG, Gui-Zhi-Fu-Ling-Wan ; 桂枝茯苓丸) and Sairei-to (SRT, Chai-Ling-Tang ; 柴苓湯) are shown in Table I. The ingredients of each Kampo medicine were mixed with 600 ml of water, and the whole was boiled until the volume was reduced to 300 ml.

Animals and treatment : Female MRL Mp-lpr/lpr mice (6 weeks old) were purchased from CREA Japan Co. Ltd. (Tokyo, Japan). Male C3H/He mice (6 weeks old) and male ICR mice (6 weeks old) were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan). The animals were housed under controlled conditions for lighting schedule (12 hrs of light : 12 hrs of darkness) and temperature (25±1°C). They had free access to a standard diet.

Chemicals : The chemicals used in this experiment were as follows : thioglycollate medium (Eiken Chemical Co., Ltd., Osaka, Japan), peroxidase anti-peroxidase (Lipsshaw/Immunon Company, Michigan, USA), Nonidet P-40 (Iwai Kagaku Co., Ltd., Tokyo, Japan), *o*-phenylenediamine dihydrochloride (OPD) (Tokyo Kasei Company, Tokyo, Japan), glucose oxidase anti-glucose oxidase complex (GAG) (ICN Immuno Biologicals, Lisle, IL), horseradish peroxidase and 4-aminoantipyrine (4-AAP) (Sigma Chemical Co., St Louis, MO) and collagenase (Nitta gelatin Co., Ltd., collagenase S-1 from *Streptomyces parvulus subsp. citrinus*. Osaka, Japan). These chemicals were obtained from each company indicated in parenthesis.

In vivo immune complexes clearance assay : The clearance of immune complexes from the circulation of mice was measured as previously described.[7] In brief, GAG which was a model of immune complexes was injected into the tail vein of mice (8 μl/g body weight). After that, 50 μl of blood was obtained from the retro-orbital plexus exactly at 6, 10, 14, and 18 min after GAG injection. Each blood sample was mixed with an equal volume of heparin solution (10 U/ml in PBS), and then centrifuged. The supernatants were assayed for glucose oxidase activity. Twenty μl of the supernatant was transferred into a microtitre well to which 100 μl of distilled water was added, and allowed to stand for 15 min. One hundred μl of reaction fluid (10 U of horseradish peroxidase, 1 g of glucose, 200 μl H_2O saturated phenol and 5 mg of 4-AAP in 10 ml of distilled water) was added to each, and the mixture incubated at room temperature. Absorbance was measured at 10 and 30 min later using a microtitre plate reader (TOSOH, MPR A4) at 492 nm. To allow for variation in background absorbance values, the glucose oxidase activity was expressed as follows ; OD 492 nm = (OD 492 nm) 30 min - (OD 492 nm) 10 min. The value of the first blood sample (taken at 6 min) was regard as 100 %. The clearance rate was calculated by linear regression analysis, and the results were expressed as half-life of GAG from the circulation (T 1/2).

In vitro immune complexes binding assay : The binding of immune complexes to macrophages was measured as previously described.[8] Briefly, C3H/He mice were injected with 2 ml of thioglycollate medium intraperitoneally. After 96 hours, macrophages were obtained from the peritoneal cavity. Macrophage density was adjusted to 1×10^6 cells per ml with RPMI 1640 medium. A 200 μl aliquot of cell suspension was added with 20 μl of a test solution, and was cultured in 96 wells microplate for 20 hours at 37°C under 5 % CO_2 condition. Each lyophilized sample was dissolved in normal saline to make a test solution. After incubation, each well was washed with 200 μl of phosphate buffered-saline (PBS) once. PAP was used in this study as a model immune complexes. A100 μl aliquot of PAP (0.5 μg/ml) was added to each well, and incubated for 4 hours at 4°C. Each well was washed with 200 μl of PBS four times and 50 μl of Nonidet P-40 was added as a detergent. A 200 μl aliquot of OPD (4 mg/ml containing 0.015 % H_2O_2), a substrate of peroxidase, was added to each well. After 15 minutes, reaction was stopped with 50 μl of 3M hydrochloride. Optical density of the well was measured at 492 nm usig micro-plate reader (TOSOH., MPR A4). The binding of PAP to macrophage was expressed as the optical density.

Carbon clearance assay : Carbon clearance assay was performed according to the procedure of Sljivic[9] with a slight modification. Briefly, Pelikan fount india drawing ink (518.221143 ; Hannover, Germany) was injected into the tail vein of C3H/He mice. Blood samples were obtained from the retro-orbital plexus exactly at 4, 7, 10 and 13 minutes after injection. Each sample was immediately mixed with 0.1 % sodium carbonate solution and centrifuged. Optical density of

the supernatant was measured at 660 nm. Clearance rate was calculated by linear regression analysis, and the results were expressed as T 1/2.

Preparation of Kupffer cells and in vitro immune complexes binding assay and immune complexes digestion assay : Preparation of liver cells was carried out according to the procedure of Seglen [10] with a slight modification. Briefly, C3H/He mouse was anesthetized with sodium pentobarbital. The liver was perfused with Ca-free Hanks'solution for 3 minutes and then with Hanks' solution containing 5 mM Ca and with 0.05 % (wt/vol) collagenase solution. The liver was suspended in Williams' medium E containing 10 % fetal calf serum (FCS) and the entire suspension centrifuged at 30 g for 1 minute. The supernatant which contained nonparenchymal cells was layered over 16 % metrizamide and centrifuged at 3000 g for 45 minutes. The Kupffer and endothelial cells in the metrizamide supernatant interface were recovered and washed four times. The cells were suspended in Williams medium E containing 10 % FCS, and cultured in a 96 well microplate for 3 hours at 37°C under 5 % CO_2 conditions. The adherent cells were used as Kupffer cells for the *in vitro* immune complexes binding assay and immune complexes digestion assay. *In vitro* immune complexes binding assay was carried out as described above.

To examine digestion of immune complexes, affer binding of PAP to Kupffer cells, the medium was added again to each well and cultured for 12 hours at 37°C under 5 % CO_2 conditions. After incubation, the medium was removed from each well and added 50 μl of Nonidet P-40 as detergent to stopped phagocytosis of PAP. The remaining PAP were measured by microplate reader. Immune complexes digestion was expressed as the optical density (PAP binding O.D.−remaining PAP O.D.).

Flow cytometric analysis : Resident macrophages were obtained from unmanipulated peritoneal cavity in ICR mice. Thioglycollate-elicited macrophages were also obtained from the peritoneal cavity in ICR mice 96 hours after an intraperitoneal injection with thioglycollate medium. Each sample of macrophages was suspended in RPMI 1640 medium and cultured in a plastic dish at 37°C under 5 % CO_2 condition for 2 hours. After non adherent cells were removed, the cells were cultured with the test solution for 20 hours. Adherent cells were harvested and added with fluorescein isothiocyanate (FITC)-conjugated anti-CD32/16 (FcγII/III receptor) MoAb (Pharmingen, CA, USA) and FITC-conjugated anti-CD11b (Mac-1, CR3) MoAb (Pharmingen, CA, USA) at 4°C for 1 hour, respectively. The cells were washed with PBS containing 1 % bovine serum albumin for three times and the expression of FcγII/III receptors and CR3 were determined by flow cytometer (EPICS Elite ; Coulter Electronics, FL, USA). A fluorescence histogram of at least 5000 counts was collected from each sample.

Immunofluorescence studies : Resident macrophages and thioglycollate-elicited macrophages were obtained from unmanipulated peritoneal cavity in ICR mice. The macrophages were cultured with TSS or without TSS for 20 hours. For immunofluorescence studies, macrophages were fixed with acetone at 4°C for 10 minutes and then were dried at room temperature. Immunostaining for CR3 on resident macrophages was performed in a direct method using FITC-conjugated anti-CD11b (Mac-1, CR3) MoAb (Pharmingen, CA, USA). On the other hand, immunostaining for FcγII/III receptor on thioglycollate-elicited macrophages was performed in a indirect method using rat anti-CD32/16 (FcγII/III receptor) MoAb (Pharmingen, CA, USA) followed by goat anti-rat IgG FITC-conjugate (COSMO BIO Co., LTD., Tokyo, Japan).

Preparation of the combined extract of Angelicae Radix and Atractylodis Lanceae Rhizoma : Preparation of the combined extract of Angelicae Radix (AR) and Atractylodis Lanceae Rhizoma (ALR) is shown in Fig. 1. AR (30 g) and ALR (40 g) were decocted with distilled water together and the extract was filtrated. The lyophilized extract was applied to Sephadex LH-20 to exclude tannins and polyphenols. The eluate was lyophilized again (AR+ALR fraction) and was suspended with distilled water. The suspension was dialyzed, and a low molecular weight fraction (LMW) and high molecular weight fraction (HMW) were obtained. LMW was applied to Sephadex G-25 and was fractionated (LMW1~7). Further, LMW5 was fractionated by silica gel column chromatography (LMW5-A~5-D). These fractions

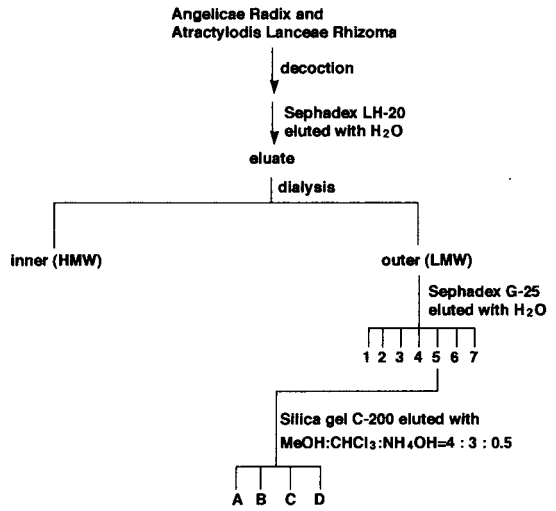

Fig. 1 Preparation of the combined extract of Angelicae Radix and Atractylodis Lanceae Rhizoma.

were carried out *in vitro* immune complexes binding assay.

Statistical Analysis : Data were analyzed by Student's *t*-test to determine significance.

Results

Effect of Kampo medicines on the clearance of immune complexes from the circulation in MRL Mp-lpr/lpr mice

Kampo medicines were administered to 10 weeks old MRL Mp-lpr/lpr mice through drinking water for 6 weeks. The daily dose of Kmpo medicines were controlled (corresponding to a 20-fold dose per kg body weight in adult humans), by regulating its concentration in relation to water consumption. After completion of the administration period, the clearance of immune complexes was measured. Significant difference was not observed in the body weight, liver weight and spleen weight between Kampo medicine-treated mice and control mice (Table II). The clearance of immune complexes in TSS-treated mice was significantly accelerated, however no significant differences were observed in Chorei-to (CRT)-, Keishi-bukuryo-gan (KBG)-and Sairei-to (SRT)-treated mice.

Effects of Kampo medicines on immune complexes binding to macrophages

After 20 hours of incubation of macrophages with Kampo medicine, immune complexes binding to macrophages were measured. The binding of immune complexes to macrophages was significantly increased by TSS treatment (Fig. 2). However, no significant differences were observed in other Kampo medicine treated macrophages.

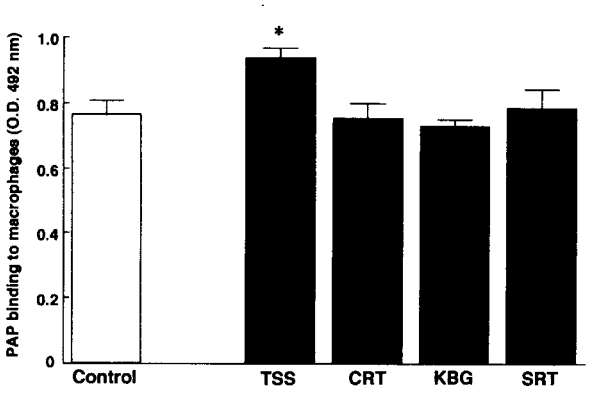

Fig. 2 Effect of Kampo medicines on binding of immune complexes to macrophages.
Each value expresses mean±S.D. (n=8)
The concentration of each sample was 500 μg/ml.
TSS, Toki-shakuyaku-san ; CRT, Chorei-to ; KBG, Keishi-bukuryo-gan ; SRT, Sairei-to
Significant difference from the control $^*p<0.01$

Table II Effect of Kampo medicines on the clearance of immune complexes in MRL Mp-lpr/lpr mice.

	Body weight (g)	Liver weight (g)	Spleen weight (g)	T 1/2 (min.)
Control	41.2±2.9	2.36±0.46	0.63±0.23	13.0±2.50
TSS	42.3±2.5	2.31±0.23	0.61±0.10	8.51±1.83*
CRT	36.0±9.1	1.87±0.54	0.46±0.23	10.6±1.88
KBG	40.2±4.4	2.22±0.29	0.64±0.15	12.1±2.20
SRT	37.2±6.5	2.15±0.43	0.58±0.30	12.6±1.94

Each value expresses mean±S.D. (n=5-7)
TSS, Toki-shakuyaku-san ; CRT, Chorei-to ; KBG, Keishi-bukuryo-gan ; SRT, Sairei-to
Significant difference from the control $^*p<0.01$

Time course of the changes in immune complexes binding to macrophages with TSS treatment

After addition of TSS, macrophages were incubated for 1 to 24 hours and immune complexes binding was measured. Immune complexes binding to macrophages was significantly increased in 12 to 24 hours (Fig. 3). Incremental binding of immune complexes to macrophages reached a plateau at 20 hours. Therefore, we decided that incubation time of macrophages with test solution would be 20 hours in the immune complexes binding assay.

Effect of TSS on immune complexes binding to Kupffer cells and digestion

Incremental binding of immune complexes to Kupffer cells was observed in TSS treated Kupffer cells (Fig. 4). After binding of immune complexes to Kupffer cells, medium was again added to wells and cultured for phagocytosis of immune complexes. The digestion of immune complexes was increased in TSS treated Kupffer cells compared with the control (Fig. 5).

Effect of TSS on the clearance of immune complexes and on carbon clearance from the circulation in C3H/He mice

TSS was administered in the drinking water for 20 days. The clearance of immune complexes from the circulation was significantly shortened in the TSS treated mice compared with control mice (Table III).

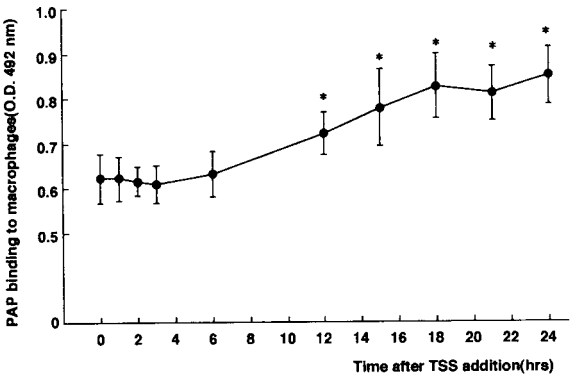

Fig. 3 Effect of Toki-shakuyaku-san on binding of immune complexes to macrophages.
Each value expresses mean±S.D. (n=8)
The concentration of each sample was 1000 μg/ml.
Significant difference from the control *$p<0.01$

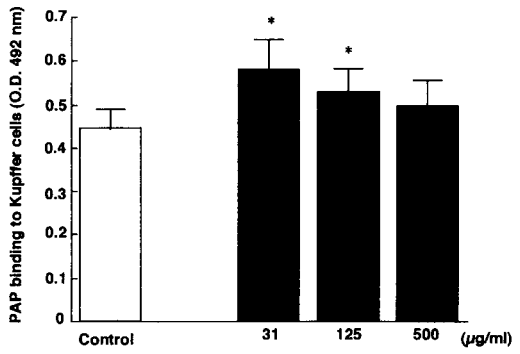

Fig. 4 Effect of Toki-shakuyaku-san on binding of immune complexes to Kupffer cells.
Each value expresses mean±S.D. (n=6-7)
Significant difference from the control, *$p<0.05$

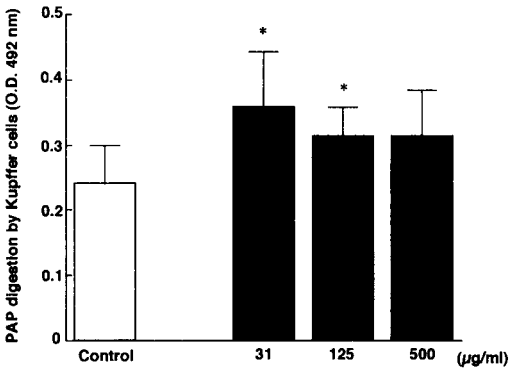

Fig. 5 Effect of Toki-shakuyaku-san on digestion of immune complexes by Kupffer cells.
Each value expresses mean±S.D. (n=6-7)
Significant difference from the control, *$p<0.05$

Table III Effect of Toki-shakuyaku-san on immune complexes clearance and on carbon clearance in C3H/He mice.

	Body weight (g)	Liver weight (g)	Spleen weight (mg)	ICs clearance T 1/2 (min.)	Carbon clearance T 1/2 (min.)
Control	27.1±0.80	1.16±0.03	77.1±7.86	8.42±0.90	10.4±2.15
TSS	27.2±1.08	1.18±0.08	76.3±11.2	7.11±0.35*	10.8±2.11

Each value expresses mean±S.D. (n=7)
TSS, Toki-shakuyaku-san ; ICs, immune complexes
Significant difference from the control *$p<0.01$

On the other hand, there was no significant difference observed in T 1/2 of carbon clearance test between TSS treated mice and control mice.

Effects of TSS on the expression of FcγII/III receptors and CR3 on the surface of macrophages

The expression of both FcγII/III receptors and complement receptor 3 (CR3) on macrophages were detected by flow cytometry. The expression of FcγII/III receptors on thioglycollate-elicited macrophages increased with TSS treatment (Fig. 6). However, TSS treatment had no effect on the expression of FcγII/III receptors on resident macrophages (data not swown). On the other hand, the expression of CR3 on TSS treated resident macrophages increased compared with control macrophages (Fig. 7). No significant change was observed in CR3 expression on

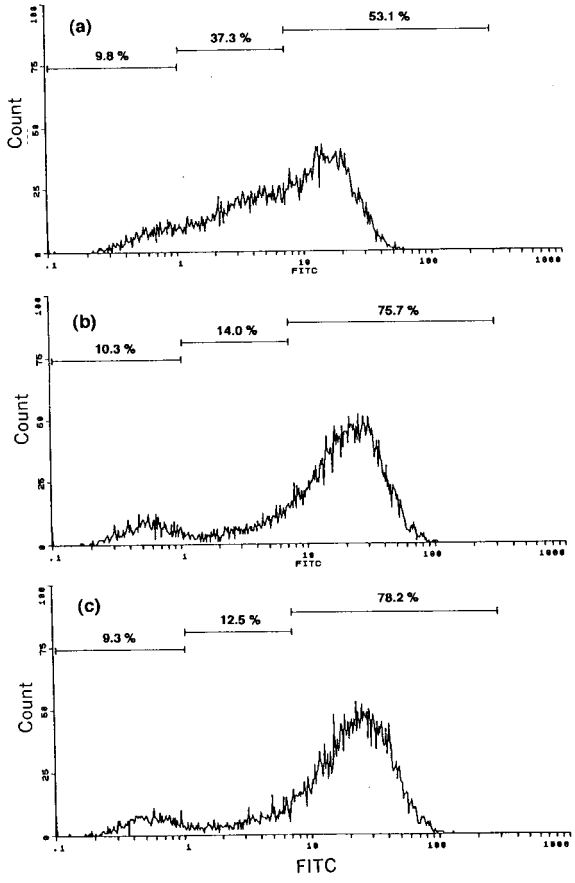

Fig. 6 Effect of Toki-shakuyaku-san on the expression of FcγII/III receptors on macrophages as detected by flow cytometry.
(a) Control, (b) Toki-shakuyaku-san treated (100 μg/ml), (c) Toki-shakuyaku-san treated (200 μg/ml)

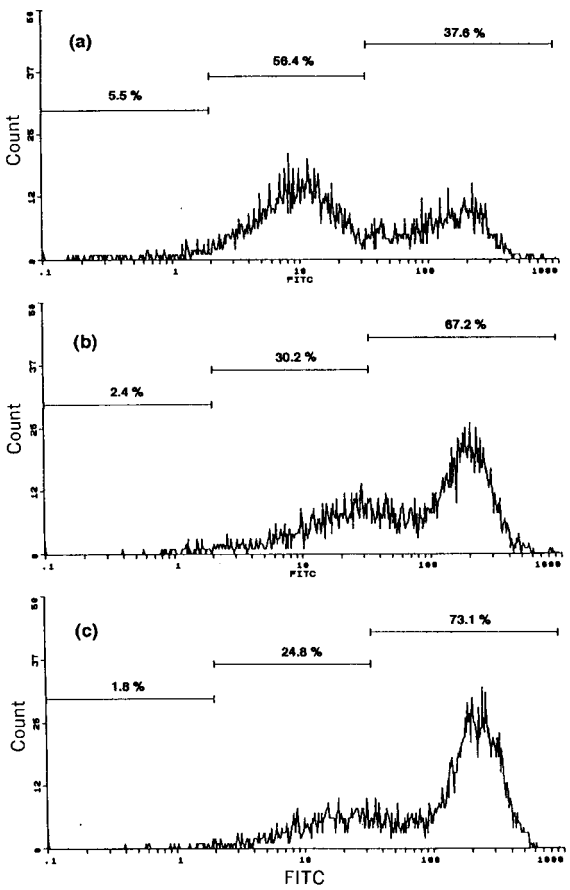

Fig. 7 Effect of Toki-shakuyaku-san on the expression of CR3 on macrophages as detected by flow cytometry.
(a) Control, (b) Toki-shakuyaku-san treated (100 μg/ml), (c) Toki-shakuyaku-san treated (200 μg/ml)

thioglycollate-elicited macrophages (data not shown).

These results were confirmed by immunofluorescence studies. The percentages of FcγII/III receptor positive cells in thiogycollate-elicited macrophages were 44 % and 20 % on TSS treatment and on control, respectively (data not shown). CR3 also increased in TSS treated resident macrophages compared with control macrophages (Fig. 8).

Effects of AR+ALR fraction, LMW fraction and HMW fraction on immune complexes binding to macrophages

The binding of immune complexes to macrophages was significantly increased in AR + ALR, LMW and HMW fractions treatment compared with control ($p<0.05$) (The value of control, AR+ALR, LMW and HMW were 0.522 ± 0.020, 0.810 ± 0.052, 0.615 ± 0.096 and 0.933 ± 0.063, respectively).

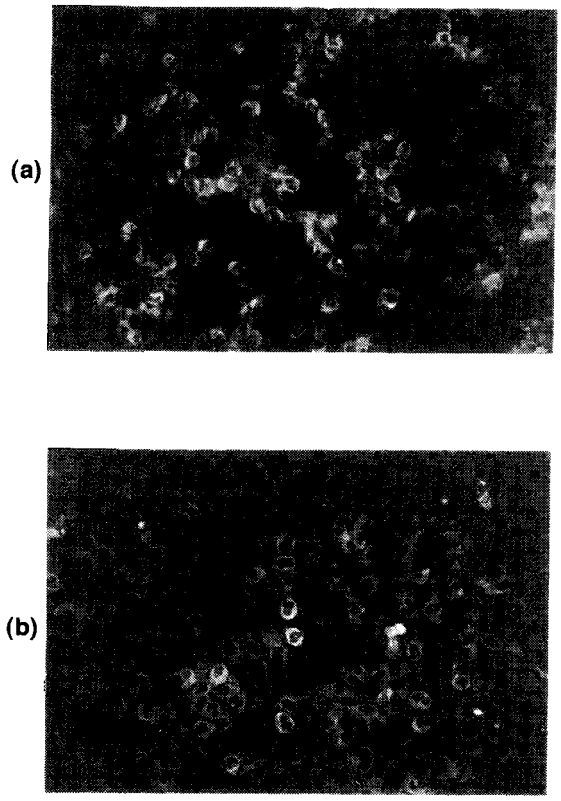

Fig. 8 Immunofluorescence studies on the expression of CR3.
(a) Control, (b) Toki-shakuyaku-san treated (100 μg/ml). ×200.

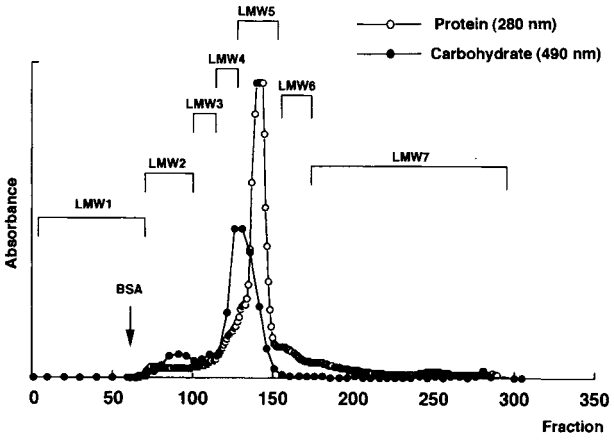

Fig. 9 Gel filtration of LMW on Sephadex G-25.
LMW, low molecular weight fraction

Effects of LMW2, 3, 4, 5, 6 and 7 fractions and LMW5-C fraction on immune complexes binding to macrophages

LMW was applied on Sephadex G-25 column

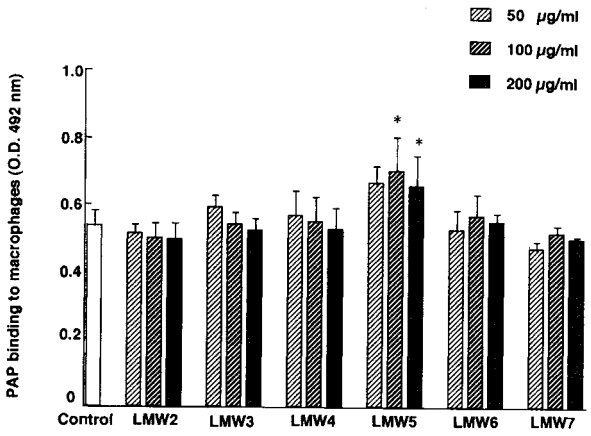

Fig. 10 Effects of LMW2-7 on immune complexes binding to macrophages.
Each value expresses the mean±S.D. (n=4)
LMW, low molecular weight fraction
Significant difference from the control, $*p<0.05$

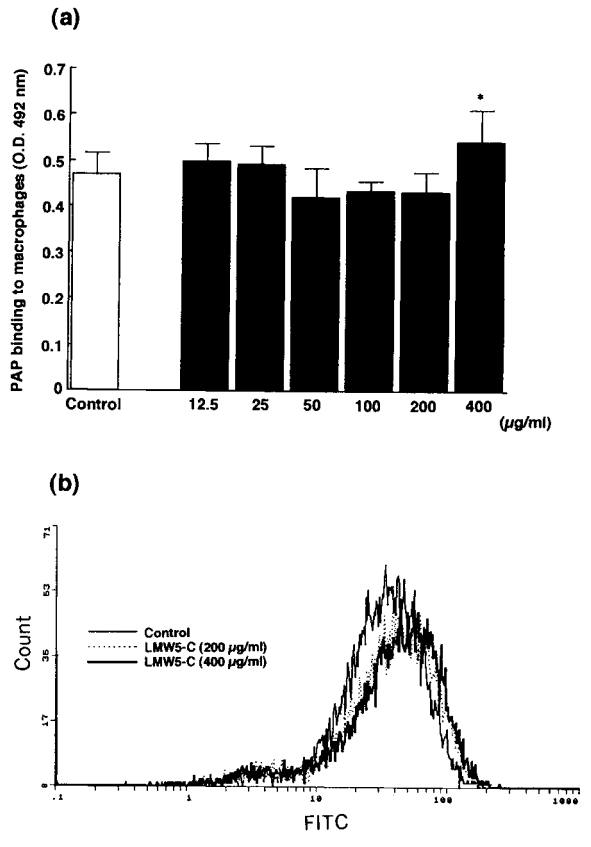

Fig. 11 Effect of LMW5-C fraction on immune complexes binding to macrophages and the expression of FcγII/III receptors.
(a) Immune complexes binding assay,
Significantly difference from the control ; $*p<0.05$ (n=8)
(b) Flow cytometric analysis

chromatography to obtain seven fractions (LMW 1~7 ; Fig. 9). The yields of each fraction were 2 (11.5 %), 3 (8.0 %), 4 (61.1 %), 5 (17.4 %), 6 (1.0 %) and 7 (1.0 %), respectively. In the result of *in vitro* immune complexes binding assay, LMW5 significantly increased the binding of immune complexes to macrophages whereas other fractions did not (Fig. 10). From the results of dialysis and gel filtration, it is speculated that the molecular weight of LMW5 was less than 3000. Furthermore, LMW5 was fractionated by silica gel column chromatography and LMW5-A, 5-B, 5-C and 5-D fractions were obtained. Increment of immune complexes binding to macrophages and of the expression of FcγII/III receptor were observed in LMW5-C only (Fig. 11).

Discussion

We previously reported that TSS increased immune complexes binding to macrophages *in vitro*.[6] In the present study, we examined whether increasing elimination of immune complexes was specific or a non-specific activation of mononuclear phagocyte system. Furthermore, we examined the effects of TSS on the expression of Fc receptors and CR3 on macrophages. In addition, we tried to separate the active component(s) of TSS by monitoring the immune complexes binding to macrophages.

The clearance of immune complexes was significantly accelerated in TSS administered MRL MP-lpr/lpr mice, and this *in vivo* result was in accord with the *in vitro* immune complexes binding assay. Further, the clearance of immune complexes was also accelerated in TSS administered C3H/He mice, however no significant change was observed in carbon clearance assay. These results indicate that TSS enhances immune complexes clearance in autoimmune disease mice and non-autoimmune strain mice, and it was suggested that enhancing phagocytosis in mononuclear phagocyte system was specific for the clearance of immune complexes. On the other hand, the clearance of immune complexes was not accelerated in Chorei-to (CRT)-, Keishi-bukuryo-gan (KBG)- and Sairei-to (SRT)-treated mice. In general, these Kampo medicines were used in the remedy of autoimmune diseases. We previously reported that Sairei-to improved the supression of the clearance of immune complexes caused by steroid administration.[11] These findings suggested that the effects of Kampo medicines against the clearance of immune complexes differed in assay methods. In addition, it was considered that the action mechanisms of these Kampo medicines might differed from TSS in the therapy of autoimmune diseases, and further investigation is needed.

Immune complexes are commonly eliminated by reticuloendothelial cells including Kupffer cells, spleen macrophages and the other tissue macrophages. Therefore, the binding of immune complexes to Kupffer cells and the digestion of immune complexes by Kupffer cells were examined. It was observed that both binding and digestion increased in TSS treated Kupffer cells, while no significant difference was shown in immune complexes binding between TSS treated spleen macrophages and control (data not shown). Although we did not examine the effects of TSS on immune complexes binding to other tissue macrophages, the results obtained here indicate the action of TSS is to increase immune complexes binding and digestion through Kupffer cells specifically.

In general, Fc receptor and complement receptor are considered to have an important role for the clearance of immune complexes. The expression of FcγII/III receptors increased in thioglycollate elicited-macrophages treated with TSS. On the other hand, the expression of CR3 also increased in resident macrophages treated with TSS but not thioglycollate-elicited macrophages. Likewise, it was observed that FcγII/III receptors and CR3 increased in TSS treated macrophages in immunofluorescence studies. Some authors have shown[12, 13, 14] that injection of the peritoneal cavity with a sterile inflammatory agent (*e.g.* thioglycollate, caseinate, peptone) differentiated the resident macrophages into responsive macrophages, and the chain of events, cell membrane markers, capacities and functions of resident macrophages were changed. Starobinas *et al.* reported[15] that phagocytosis of immune complexes through Fcγ receptors and CR3 increased in thioglycollate elicited-macrophages. Abel *et al.* reported[16] that non-specific phagocytosis of fluorescent latex microbeads by macrophages increased in thioglycollate elicited-

macrophages. However, as TSS increased Fc receptor expression on responsive macrophages and increased CR3 expression on resident macrophages, it should be considered that these are different responses of the stages of cell differentiation.

It is revealed that one of the action mechanisms of TSS on immune complexes clearance was the increasing expression of Fc receptors and CR3 on macrophages.

Next, we tried to determine the active component (s) of TSS. Previous studies in our laboratory have shown that the action of TSS enhanced immune complexes binding to macrophages was mainly contributed by Angelicae Radix (AR), and the preparation of the combined extract of AR and Atractylodis Lanceae Rhizoma (ALR) had an important role on the appearance of the enhancing activity.[6] Therefore, we tried to prepare from the combined extract of AR and ALR. The combined extract of AR and ALR was dialyzed, and LMW and HMW fractions were obtained. Both LMW and HMW increased immune complexes binding. In the course of our studies, it was already known[17,18] that several polysaccharides from Kampo medicines and AR had anti-complementary activities and mitogenic activities. According to these reports, the enhancing activity of HMW from AR and was also supposed to be polysaccharides. Therefore, the present investigation was focused on LMW fraction. LMW was further fractionated by Sephadex G-25 chromatography to obtain seven fractions. An increase in the binding of immune complexes to macrophages was observed in fraction 5 (LMW5) only. LMW5 was further fractionated by silica gel column chromatography and LMW5-C which increased immune complexes binding to macrophages and the expression of FcγII / III receptor was obtained. Although the LMW5-C fraction has not been purified, it is believed that enhanced clearance of immune complexes was derived in part from these components. Studies on the active components are underway in our laboratory.

In conclusion, we present that one of the mechanisms of action of TSS on the immune system and enhancement of immune complexes clearance is due to increased immune complexes binding to macrophages *via* augmentation of FcγII/III receptors and CR3 expression. The details of action and active components will require further investigation.

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和文抄録

自己免疫疾患の病態形成に免疫複合体が関与していることに着目し，免疫複合体除去能に対する種々の漢方薬の影響について検討を行なった。その結果，当帰芍薬散は自己免疫疾患モデルマウスおよび近交系マウスの *in vivo* での免疫複合体除去能を亢進したが，網内系細胞の非特異的貪食能測定系であるカーボンクリアランスは亢進しなかった。また当帰芍薬散は肝臓のクッパー細胞の免疫複合体結合能および消化能を増加した。一般に免疫複合体は，マクロファージのFcおよびC3受容体を介して効率的かつ速やかに除去されることから，マクロファージ細胞表面のFcおよびC3受容体発現量を，フローサイトメーターおよび免疫螢光法を用いて測定した。その結果，当帰芍薬散をマクロファージに作用させることにより，FcおよびC3受容体の増加が観察された。当帰と蒼朮の混合熱水抽出物から透析により得られた高分子画分および低分子画分について検討したところ，両者でマクロファージの免疫複合体結合能は増加した。低分子画分は精製を行ない7分画に分けたところ，LMW5のみに活性が観察された。更にLMW5より精製されたLMW5-Cは，免疫複合体結合能およびFc受容体の発現を増加した。これらの結果より，当帰芍薬散の *in vivo* での免疫複合体除去能亢進の作用機序は，網内系細胞の非特異的活性化によるものではなく，マクロファージのFcおよびC3受容体を増加させることによると考えられ，多糖類をはじめとする高分子画分のみではなく，低分子画分にも活性成分が存在することが示された。

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