Original 和漢医薬学会誌 **10**, 28－33, 1993

Effects of Kampo medicine on immune complexes binding to macrophages *in vitro*
－Blend effects of components of Toki-shakuyaku-san－

Koji IIJIMA,* Morihisa TANAKA, Kazuo TORIIZUKA and Jong-Chol CYONG

Oriental Medicine Research Center of the Kitasato Institute

(Received November 12, 1992. Accepted March 8, 1993.)

Abstract

The effects of Toki-shakuyaku-san (TSS) and each ingredient on the binding of immune complexes to macrophages were demonstrated. To elucidate the activity, the investigation carried out the peroxidase anti peroxidase complex (PAP), as model immune complex, binding to macrophage *in vitro*. Samples were prepared from decoction. To exclude tannins and polyphenols, the decoctions were applied to Sephadex LH-20. The eluates with water were lyophilized to get sample extracts. Decoctions of TSS and crude drugs were applied to Sephadex LH-20 to exclude tannins and polyphenols, and liophlized to get the test samples. Immune complexes binding was significantly enhanced by the TSS extract. To clarify the role of the complex of six ingredients ; we have examined the activity of individual ingredients, and "minus one crude drug prescriptions". No significant changes were observed in the individual components. The enhancing activity of TSS has still remained in "minus one crude drug prescriptions" except the TSS minus Angelicae Radix extract. Although no activity was detected in a simple mixture of Angelicae Radix extract and TSS minus Angelicae Radix extract, the enhancing activity was obtained in the combined extract of Angelicae Radix and Atractylodis Lanceae Rhizoma. The results obtained in this experiment indicated the following ; TSS enhanced the ability of the immune complexes binding to macrophages ; this action of TSS was mainly contributed by Angelicae Radix, and the combination of Angelicae Radix and Atractylodis Lanceae Rhizoma had an important role on the appearance of the enhancing activity of TSS.

Key words Toki-shakuyaku-san, immune complexes, macrophages, Angelicae Radix, Atractylodis Lanceae Rhizoma, blend effects.

Abbreviations ALI, Alismatis Rhizoma ; ALR, Atractylodis Lanceae Rhizoma ; AR, Angelicae Radix ; ClCs, circulating immune complexes ; CR, Cnidii Rhizoma ; EIC, enzymatic immune complexes clearance ; H, Hoelen ; MPS, mononuclear phagocytic system ; OPD, O-phenylenediamine dihydrochloride ; PAP, peroxidase anti peroxidase complex ; PBS, phosphate buffered saline ; PR, Paeoniae Radix ; SLE, systemic lupus erythematosus ; TSS, Toki-shakuyaku-san ; Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San), 当帰芍薬散.

Introduction

It has been suggested that deposition of immune complex to tissues is one of the important pathogenesis of autoimmune diseases. Immune complexes are generally detected in the circulation in systemic lupus erythematosus (SLE) patients[1,2] and they have been considered to be a causative agent of glomerulonephiritis, vasculitis and skin diseases.[3,4]

Recently, some clinical reports[5] have indicated that the traditional Japanese (Kampo) medicines improved the patients with autoimmune

*〒108 東京都港区白金 5-9-1
北里研究所附属東洋医学総合研究所　飯島宏治
5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **10**, 28－33, 1993

diseases. And also, during our course of study on the effects of Kampo medicines on the immune system, we revealed[6] that the clearance of immune complexes from the circulation of autoimmune disease model mice (*in vivo* enzymatic immune complex clearance assay : *in vivo* EIC assay) was significantly increased by Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San in Chinese ; *Pulvis paeoniae et angelicae*) treatment.

Toki-shakuyaku-san (TSS) is one of the most common Kampo prescriptions. TSS is one of Kampo formulations frequently applied to gynaecological disorders. According to classic Kampo textbooks, TSS has been shown to improve ovarin dysfunction, and has been empirically used as a remedy for amenorrhea, luteal phase dyafunction, and anovulatory syndrome. TSS is a combination of six medicinal plants ; Angelicae Radix (AR), Cnidii Rhizoma (CR), Paeoniae Radix (PR), Hoelen (H), Atractylodis Lanceae Rhizoma (ALR), and Alismatis Rhizoma (ALI). According to our *in vivo* EIC assay,[6] this medicine improved the clearance of immume complexes, however no effect was observed in each ingredient of TSS. This finding suggested that the combination of medical plants might have an important role on the activity of this prescription. Therefore, our next endeavour was focused on examining if Toki-shakuyaku-san has potential effects on *in vitro* immune complexes binding to macrophages, and to clarify the interaction of ingredients.

Materials and Methods

Crude drugs : Crude drugs (medical plants) were purchased from Uchida Wakan-Yaku (Tokyo, Japan). Toki-shakuyaku-san was prepared by decoction. Six herbs (Angelicae Radix, from Nara, Japan, 3.0 g ; Cnidii Rhizoma, from Hokkaido, Japan, 3.0 g ; Paeoniae Radix, from Nara Japan, 4.0 g ; Hoelen, from Korea, 4.0 g ; Atractylodis Lanceae Rhizoma, from Hubei, China, 4.0 g ; Alismatis Rhizoma, from Guizho, China, 4.0 g) were mixed with 600 ml of distilled water, and the whole was boiled until the volume was reduced to 300 ml. The decoction was filtrated immediately. The residue was added to 600 ml of water, and boiled again. This procedure was repeated twice. The filtered hot water extracts were combined, and lyophilized. To exclude tannins and polyphenols, the extract was suspended with 20 ml of water, and applied to Sephadex LH-20 (10 ml), and eluted with 50 ml of water. The eluate was lyophilized again. To evaluate the interaction of each ingredient, we also made a "single crude drug" decoction and "minus one crude drug" decoction in the same way.

The weight of the lyophilized extracts were as follows : the yield (%), calculated by the weight of TSS extract was 100 %, is given in parentheses ; TSS, 6.131 g (100 %) : AR, 1.457 g (23.8) ; CR, 0.959 g (15.6) ; PR, 1.361 g (22.2) ; H, 0.085 g (1.4) ; ALR, 1.459 g (23.8) ; ALI, 0.538 g (8.8) ; TSS−AR, 4.655 g (75.9) ; TSS−CR, 5.203 g (84.9) ; TSS−PR, 4.757 g (77.6) ; TSS−H, 6.124 g (99.8) ; TSS−ALR, 4.451 g (72.6) ; TSS−ALI, 5.627 g (91.8).

Animals : Male C3H He/J mice (6 weeks of age) used in this experiment were purchased from Nihon SLC Co. Ltd (Hamamatsu, Japan). A lighting schedule (12 hours of light ; 12 hours of darkness) and controlled temperature were used. They had free access to standard diet and drinking water.

Chemicals : Thioglycollate medium were obtained from Eiken Chemical Co., Ltd. (Osaka, Japan). Neat goat peroxidase anti peroxidase (PAP) were obtained from Lipshaw / Immunon Company (Michigan, USA). Nonidet P-40 were obtained from Iwai Kagaku Co., Ltd. (Tokyo, Japan). O-phenylenediamine dihydrochloride (OPD) were obtained from Tokyo Kasei Company (Tokyo, Japan).

Measurement of immune complexes binding : The binding of immune complexes to macrophages were measured as previously described.[7] In brief, mice were injected with 2 ml of thioglycollate medium intraperitoneally. After 96 hours of injection, 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

However, in the case of "minus one crude drug" decoction, only "TSS−Angelicae Radix" decoction represented the significant decrease of PAP binding compared to TSS treatment. Other "minus one crude drug" decoctions (−Cnidii Rhizoma, −Paeoniae Radix, −Hoelen, −Atractylodis Lanceae Rhizoma, and −Alismatis Rhizoma) still had the same PAP binding activity as TSS (Fig. 2).

Furthermore, we tested the effects of the combination. The results were showed in Fig. 3. The mixture of Angelicae Radix etract and TSS −Angelicae Radix extract no effect was observed on PAP binding. These observations strongly suggested that Angelicae Radix contributed to the enhancement activity of TSS on binding of macrophages to the immune complexes. Also, its combination with other crude drugs might be important to express the activity. Therefore, the next experiment was designed to examine if the blend of two crude drugs decoction has an enhancing activity on the binding of macrophage.

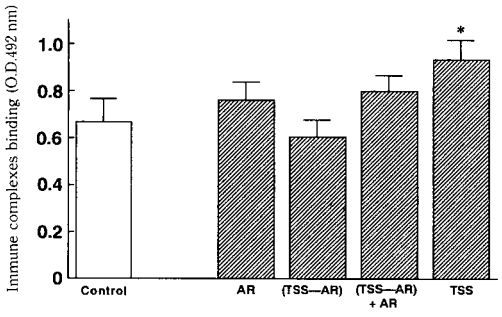

Fig. 3 Effects of the mixture of "Angelicae Radix extract and Toki-shakuyaku-san minus Angelicae Radix extract" on immune complexes binding to macrophages.
TSS : Toki-shakuyaku-san, AR : Angelicae Radix, (TSS - AR) : Toki - shakuyaku - san minus Angelicae Radix
Sample concentration was calculated as described in "the test".Each value expresses the mean±S.D. (n=8) Significant difference from the control, $*p<0.001$

Effect of the combination of two crude drugs, Angelicae Radix and other crude drugs on immune complexes binding

Angelicae Radix and other component of TSS were decocted together, and the effect on immune complexes binding were assayed. The weight of "combination of two crude drugs" extracts were as follows, the yield is given in parentheses ; TSS, 6.131 g (100 %) ; AR+CR, 2.749 g (44.8) ; AR+PR, 2.486 g (40.5) ; AR+H, 1.352 g (22.1) ; AR+ALR, 2.700 g (44.0) ; AR+ALI, 1.891 g (30.8). Each sample was dissolved with normal saline to an adequate concentration, which gave the same concentration of TSS (1000 μg / ml, final), calculated by the yeild. As shown in Fig. 4, macrophages treated with the extract of Angelicae Radix and Atractylodis Lanceae Rhizoma significantly enhanced immune complexes binding. No signifivant changes of immune complexes binding were observed in other combinations.

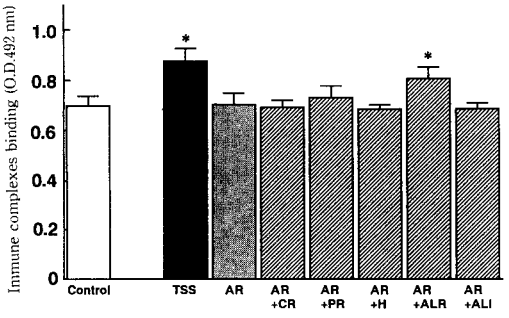

Fig. 4 Effects of combination of two crude drugs on immune complexes binding to macrophages.
TSS : Toki-shakuyaku-san, AR : Angelicae Radix, CR : Cnidii Rhizoma, PR ; Paeoniae Radix, H : Hoelen, ALR : Atractylodis Lanceae Rhizoma, ALI : Alismatis Rhizoma
Sample concentration was calculated as described in "the text". Each value expresses the mean±S.D. (n=8) Significant difference from the control, $*p<0.001$

Discussion

When exposed to antigens, an individual responds by producing specific antibodies, which combine with the inciting antigens to form immune complexes. This response is well designed to eliminate and/or neutralize antigens, and is beneficial for the host. However, the process is sometimes accompanied with a variety of inflammatory reactions, known as the Type III reaction.[8-10]

37°C under 5 % CO_2 condition. Each lyophilized sample was dissolved with 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 complex. A 100 μl 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 of OPD (4 mg/ml containing 0.015 % H_2O_2), as substrate of peroxidase was added to each well. After 15 minutes, reaction was stopped with 50 μl of 3 M hydrochloride. Optical density of the well was measured at 492 nm using micro-plate reader (TOSOH., MPR A4). The binding of PAP to macrophage was expressed at the optical density.

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

Results

Effect of Toki-shakuyaku-san on immune complexes binding

Twenty hours incubtion with Toki-shakuyaku-san resulted in an increase of PAP binding in a dose depending manner. As shown in Fig. 1, the binding was significantly increased at the concentration of 500 μg/ml and 1000 μg/ml ($p<0.05$, and $p<0.001$, respectively).

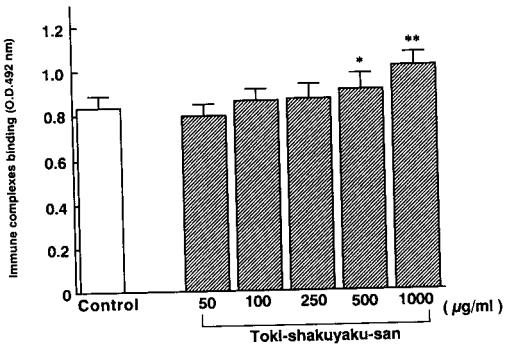

Fig. 1 Effect of Toki-shakuyaku-san on immune complexes binding to macrophages. Each value expresses the mean±S.D. (n=8) Significant differences from the control, $*p<0.05$, $**p<0.001$

Effects of "single crude drug" decoctions and "minus one crude drug" decoctions on immune complaxes binding

Table I shows the results of the immune complexes binding treated with single crude drug decoctions. The concentration of each sample was converted the same as that of TSS (1000 μg/ml, final concentration), calculated by the yield of each extract. No significant change was observed.

Table I Effect of single crude decoction on immune complexes binding to macrophages.

Herbs	Immune complexes binding (O.D.492 nm)
Control	0.686±0.038
Angelicae Radix	0.704±0.025
Cnidii Rhizoma	0.663±0.049
Paeoniae Radix	0.693±0.014
Hoelen	0.686±0.032
Atractylodis Lanceae Rhizoma	0.699±0.038
Alismatis Rhizoma	0.691±0.047
Toki-shakuyaku-san	0.850±0.038*

The immune complexes bindings were determined as described in "Materials and Methods". Sample concentraion was described in "the text". Each value expresses the mean±S.D.(n=8). Significant difference from the control, $*p<0.001$

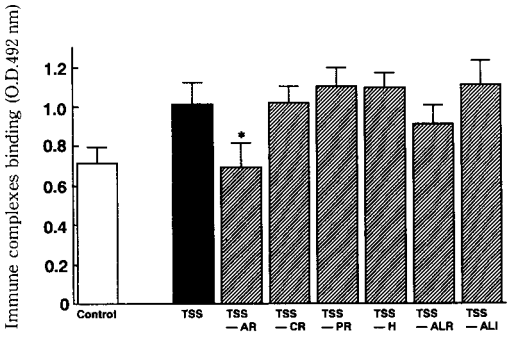

Fig. 2 Effects of "minus one crude drug" decoctions on immune complexes binding to macrophages.
TSS : Toki-shakuyaku-san, AR : Angelicae Radix, CR : Cnidii Rhizoma, PR : Paeoniae Radix, H : Hoelen, ALR : Atractylodis Lanceae Rhizoma, ALI : Alismatis Rhizoma Sample concentration was calcuated as described in "the text". Each value expresses the mean±S.D. (n=8) Significant difference from Toki-shakuyaku-san represent, $*p<0.001$

Circulating immune complexes (CICs) are commonly detected in many diseases, *i.e.* systemic lupus erythematosus (SLE),[1,2] neoplastic diseases,[11] hepatitis,[12] asthma,[13] Behcet's diseases,[14] rheumatoid arthritis.[15] Recently, adhesion of immune complexes to tissues has been considered to be a pathogenesis of autoimmune diseases, and to be a causative agent of glomerulonephiritis, vasculitis and skin diseases.[3,4]

In the course of our study of the Kampo medicines on the immune system, we revealed that the immune complex clearance *in vivo* experiment was significantly improved by TSS treatment.[6] The amount of glucose oxidase anti-glucose oxidase complexes, as a model immune complex, in the circulation was decreased in the TSS treated group. This result is thought to be achieved by the complexes binding to Fc receptors of the mononuclear phagocytic system (MPS) cells. Therefore, this experiment was designed to confirm the action of TSS on the immune complexes binding to macrophages using *in vitro* assay.

The binding ability of PAP to macrophages was enhanced in dose depending on the concentrations of TSS as shown in Fig. 1. it was reported that a tannin-rich fraction enhanced the non-specific binding of CICs to macrophages.[7] Nishizawa *et al.*[16] isolated the gallotannins (polygalloylglucose) from *Paeonia albiflora,* one of the ingredients of TSS, using Sephadex LH - 20 column chromatography. The samples tested in this experiment were prepared from the decoctions and treated with Sephadex LH-20 in a similar manner to that report,[7] to expect to exclude tannins and polyphenols. The eluates were checked with ferric chloride solution for negative. Therefore, the effects of TSS were not through the non-specific binding effects of these components. This result agrees with the *in vivo* observations,[6] and suggests that TSS may facilitate the ability of Fc receptors of the MPS cells bind to immune complexes and consequently enhance the CICs clearance *in vivo.*

Secondly, the experiment was designed to clarify the active components of this prescription. No enhancing activity was detected in each ingredient of TSS (Table I). However, when tested "minus one herb prescription", the enhancing activity disappeared in "TSS−Angelicae Radix" (Fig. 2). The enhancing activity was not obtained with a simple combination of "Angelicae Radix" extract and "TSS − Angelicae Radix" extract (Fig. 3). In this experiment, we adjusted the concentrations of samples the same as that of TSS, calculated by the yield of each sample, because this experiment was designed to evaluate the contribution of each ingredient on the enhancing effects of TSS. These results indicate the following ; (1) the enhancing action of TSS was mainly contributed to by Angelicae Radix, (2) the appearance of this action required the combination and decoction of Angelicae Radix and other crude drug(s) together.

The effects of combination of two crude drugs were then assayed. As shown in Fig. 4, the enhancing activity was observed only in the combination of Angelicae Radix and Atractylodis Lanciae Rhizoma. Since the enhancing activity of "TSS − Atractylodis Lanciae Rhizoma" had a tendency to decrease (Fig. 2), these results seemed to coincide with each other. The active component(s) and action mechanisms are still unclear. Studies on the active component(s) have been underway in our laboratory, but the details also needed to investigated farther.

Autoimmune disorders has emerged in the past decade as a major public health problem.[17] The etiology of the disorders has progressed, but at present there is little cure. Considerable efforts have gone into devising therapeutic strategies which might restore function in these systems. The results demonstrated here indicate the possibility of the prescription and/or crude drug(s) may have potential therapeutic effects for treating patients with autoimmune diseases.

Acknowledgement

This investigation was supported in part by Tokyo Metropolitan Health Grant, and a grant-in-aid for scientific research of Kampo medicine from Tsumura and Co., Ltd..

和文抄録

免疫複合体結合能に対する当帰芍薬散の作用について *in vitro* で検討を行った。マクロファージに当帰芍薬散を作用させることにより，免疫複合体結合能の亢進が観察された。当帰芍薬散中の構成生薬単味についても同様の検討を行ったが，作用は認められなかった。一方，一味抜きの当帰芍薬散を作成し検討した結果，当帰抜きの処方で免疫複合体結合能の亢進作用の消失が観察された。当帰芍薬散の免疫複合体結合能の亢進作用発現には当帰が寄与しているものと考えられた。また，当帰抜き処方のエキスに当帰エキスを後から加えただけでは亢進作用は認められず，当帰と他の構成生薬を一緒に煎じることが，活性発現に意義をもつものと推定された。そこで，当帰と他の構成生薬の二味の組み合わせを作り，その煎液について検討を行った結果，当帰と蒼朮の煎液エキスに作用が認められた。これらの結果より，当帰芍薬散の免疫複合体結合能の亢進作用発現には当帰と蒼朮の組み合わせと，この二味を同時に煎じることが関与していることが示され，漢方方剤煎出の薬理学的意義を裏付けるものと考えられた。また当帰芍薬散は，古くより駆瘀血剤として用いられてきたが，今回新たに免疫複合体除去能の亢進作用を有し，自己免疫疾患の治療薬としての可能性をもつことが示された。

References

1) Koffler, D.: Immunopathogenenesis of systemic lupus erythematosus. *Ann. Rev. Med.* **25**, 149, 1974.
2) Tan, E.M., Schur, P.H., Carr, R.I. and Kunker, H.G.: Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J.Clin. invest.* **45**, 1732, 1966.
3) Haakenstad, A.O. and Mannik, M.: The biology of immune complexes. In "Autoimmunity. genetic, immunologic, virologic, and clinical aspects" (Ed. by Talal, N.) Academic Press, San Dieqo, CA, pp.277-360, 1977.
4) Eilat, D.: Cross-reactions of anti-DNA antibodies and the central dogma of lupus nephritis. *Immunol. Today* **6**, 123, 1985.
5) Matsushita, K., *et al* : Collagen disease and immunological disorders. *J.Traditional Sino - Japanese Med.* **12** (Suppl.), 194-236, 1991 (in Japanese).
6) Iijima, K., Tanaka, M., Toriizuka, K. and Cyong, J-C.: Effects of Kampo Medicines on the Clearance of Circulating immune Complexes in Mice. *J. Ethnopharm.* (submitted)
7) Tanaka, M., Matsumoto, T., and Cyong, J-C.: Effects of Rhei Rhizoma on immune complex clearance *in vitro* I. *J.Med. Pharm.Soc. WAKAN - YAKU* **4**, 1-7, 1987.
Cyong, J.-C., Ishihara, A., Matsumoto, T., Tanaka, M. and Otsuka,Y.: Effect of Rhei Rhizoma on immune complex III. *J. Med. Pharm. Soc. WAKAN-YAKU* **2**, 544-545, 1985.
8) Ralph, C.W.Jr.: Connective-tissue diseases. In "Immune Complexes in Clinical and Experimental Medicine." A Commonwealth Fund Book, Harvard University Press, Cambridge, Massachusetts and London, pp 195-249, 1980.
9) Theofilopoulos, A.N. & Dixon, F.J.: Immune complexes in human disease. *Am. J. Ptathol.* **100**, 530-594, 1980.
10) Kasukawa, R.and Ohara, M.: Clinical significance of immune complex. *Clin. Immunol.* **15**, 8-17, 1983.
11) Teshima, H., Wanebo, H., Pinsky, C.and Day, N.K.: Circulating immune complexes detected by ^{125}I-C1q deviation tast in sera of cancer patients. *J.Clin.Invest.* **59**, 1134-1142, 1977.
12) Thomas, H.C., Villiers, D., Plotter, B., Hodgson, H., Jain, S., Jewell, D.P.and Sherock, S.: Immune complexes in acute and chronic liver diseases. *Clin. Exp. Immunol.* **31**, 150-157, 1978.
13) Stendardi, L., Delespesse, G. and Debisschop, M.J.: Circulating immune complexes in bronchial asthma. *Clin. Allergy* **10**, 405-411, 1980.
14) Gupta, R.C., O'duffy, J.D., Macduffie, M. and Jordon, R. E.: Circulating immune complexes in active Behcet's disease. *Clin. Exp. Immunol.* **34**, 213-218, 1978.
15) Aguado, M.T.and Theofilopoulos, A.N.: Immune complexes in human and experimental disease. In "Immunology of Rheumatic Disease" (ed.by Gupta, S.& Talal, N.), pp 493-513, Plenum Medical Book, New York, 1985.
16) Nishizawa, M., Yamagishi, T., Nonaka.G.and Nishioka, I.: Structure of gallotannins in Paeoniae Radix. *Chem. Pharm. Bull.* **28**, 2850-2852, 1980.
17) Tanimoto, K., *et al.*: Systemic lupus erythematosus. *Internal Medicine* **65**, 851-855, 1990 (in Japanese).