Effects of Kampo medicines on the clearance of immune complexes from the circulation of prednisolone treated mice

Koji IIJIMA,* Morihisa TANAKA, Tsukasa MATSUMOTO, Yasuro KAWAKUBO, Kazuo TORIIZUKA and Jong-Chol CYONG

Oriental Medicine Research Center of the Kitasato Institute

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Abstract

The present study was undertaken in an attempt to evaluate the effects of the Kampo medicines on dysfunction of the mononuclear phagocytic system (MPS). To estimate the function of MPS, this investigation was carried out *in vivo* enzymatic immune complex clearance assay (EIC assay) and carbon clearance assay.

First, we will clarify that the prednisolone administration causes the suppression of MPS in mice, and decreases the clearance of immune complexes (ICs). Second, we will present the comparative study of three Kampo prescriptions (Sho-saiko-to, Gorei-san, Sairei-to) on clearing ICs using prednisolone treated mice. The decreased clearance of ICs in prednisolone treated mice was significantly improved in the Sairei-to treatment group but not in the other two groups. In carbon clearance assay, no effect was observed in the Sairei-to treatment group. It was confirmed in the histological study that the injury of the thymus in prednisolone treated mice was improved by Sairei-to administration. These observations indicated that the treatment of Sairei-to facilitated the Fc-receptor dependent clearance and have a possibility of reducing the adverse reaction of steroid treatment.

Furthermore, this Kampo prescription may be a potentially useful therapy for autoimmune diseases.

Key words Sairei-to, circulating immune complexes (CICs), mononuclear phagocytic system (MPS), Fc-receptor.

Abbreviations CICs, circulating immune complexes; EIC, enzymatic immune complex clearance; GAG, glucose oxidase anti-glucose oxidase complexes; GRS, Gorei-san; ICs, immune complexes; LPS, lypopolysaccharide; MPS, mononuclear phagocytic system; SLE, systemic lupus erythematosus; SST, Sho-saiko-to; SRT, Sairei-to; Sho-saiko-to (Xiao-Chai-Hu-Tang), 小柴胡湯; Gorei-san (Wu-Ling-San), 五苓散; Sairei-to (Chai-Ling-Tang), 柴苓湯.

Introduction

Circulating immune complexes (CICs) are commonly detected in patients with autoimmune diseases *i.e.* systemic lupus erythematosus (SLE)[1,2] and rheumatoid arthritis.[3] Recently, deposition of immune complexes (ICs) to tissues has been considered to be one of the pathogenesis of autoimmune diseases, and to be a causative agent of glomerulonephritis, vasculitis and skin diseases.[4,5] In generally, steroid or cytostatic medicine have been used for treatment of autoimmune disorders. However, a number of clinical reports have indicated that these medicines have severe adverse reactions and it was also known that administration of cortisone decreased the clearance of CICs because of the suppression of the mononuclear phagocytic system (MPS) occured.[6] Abe *et al.*[7] reported that the traditional Japanese (Kampo) medicines have the efficacy of preventing the adverse reaction of steroid and/or cytos-

*〒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

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tatic medicines. Previously, in our course of study on the effects of Kampo medicines,[8] we revealed that administration of one of Kampo prescriptions ; Sho-saiko-to, decreased ICs from the circulation in NZB / NZW F_1 mice treated with lipopolysaccharide (LPS) intraperitoneral injection. Therefore, our next endeavor was focused to examine if Kampo medicines have potential effects on the suppression of MPS caused by steroid administration and if there is the possibility of remedies for autoimmune diseases.

Materials and Methods

Crude drugs : The Kampo medicines used in this study, Sho-saiko-to (SST ; Xiao-Chai-Hu-Tang in Chinese, *Formula bupleuri minor* in Latin), Gorei-san (GRS ; Wu-Ling-San, *Pulvis quinque-medicamentorum*), and Sairei-to (SRT ; Chai-Ling-Tang, *Formula hoelen et bupleuri*) are basically plant source medicines, and each prescription was a combination of several different medicinal plants. The recipes of formulations are as follows, with the dosage given in parentheses : Sho-saiko-to (SST) ; Bupleuri Radix (7), Pinelliae Tuber(5), Scutellariae Radix (3), Zizyphi Fructus (3), Ginseng Radix (3), Glycyrrhizae Radix (2) and Zingiberis Rhizoma (0.5) ; Gorei-san (GRS) ; Alismatis Rhizoma (6), Atractylodis Rhizoma (4.5), Hoelen (4.5), Polyporus (4.5) and Cinnamomi Cortex (3) ; Sairei-to (SRT) ; Bupleuri Radix (7), Pinelliae Tuber (5), Scutellariae Radix (3), Zizyphi Fructus (3), Ginseng Radix (3), Glycyrrhizae Radix (2), Zingiberis Rhizoma (0.5), Alismatis Rhizoma (4.5), Atractylodis Rhizoma (4.5), Hoelen (4.5), Polyporus (4.5) and Cinnamomi Cortex (3). These crude drugs were purchased from Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan) and Tsumura & Co. Ltd. (Tokyo, Japan).

Preparation of Kampo prescriptions : Three Kampo prescriptions were administered to mice in the form of decoction. The procedure used for the preparation was as follows ; Combined ingredients were mixed with 600 ml of distilled water, and the whole was boiled until the volume was reduced to 300 ml.

Chemicals : Prednisolone was obtained from Sigma Chemical Co. (St. Louis, MO., USA). Glucose oxidase (EC 1. 1. 3. 4) anti-glucose oxidase complexes (GAG) were obtained from ICN Immuno Biologicals Co. (Lisle, IL., USA). Other chemical reagents were purchased from WAKO Pure Chemical Co., Ltd. (Tokyo, Japan).

Animals : The mice used in this experimentation were purchased from Nihon SLC, Co. Ltd. (Hamamatsu, Japan). The animals were housed in a lighting schedule (14 hrs of light ; 10 hrs of darkness) in controlled temperature. They had free access to standard diet.

Experimental design

1) Forty male ICR mice were divided into four groups : prednisolone (1.2, 6.0 and 30 mg/kg) was injected subcutaneously daily for 20 days beginning at 7 weeks of age. The control group received saline only. After 20 days, the clearance of ICs was measured by *in vivo* enzymatic immune complex clearance (EIC) assay.[9]

2) Fifty male C3H/He mice were divided into five groups : Four groups basically received a *s.c.* dose of prednisolone (1.2 mg/kg) daily during the experimental period. The control group received saline only. Kampo prescriptions were administered in the drinking water for 14 days. The dose was 20-fold in human dose per kg body weight, by regulating its concentration in relation to water consumption.

In vivo enzymatic immune complex clearance (EIC) assay : The clearance of ICs from the circulation of animals was measured as previously described.[9] In brief, a solution of GAG (20 μg/ml) was injected into the tail vein of the mice (8 μl/g body weight). Thereafter, blood samples were obtained from the retro-orbital plexus at exactly 6, 10, 14, and 18 minutes after the GAG injection. Each blood sample was mixed with an equal volume of heparin solution (10 U/ml in phosphate-buffered saline : Novo Industry, Copenhagen, Denmark) and then centrifuged. The resulting supernatants were measured for glucose oxidase activity using a microtitre plate reader (Titertek multiscan) at 405 nm. The glucose oxidase activity in the first blood sample was taken as the 100% value. Clearance rate was calculated by linear regression analysis, and the results were

expressed as T1/2 (half-life of GAG from the circulation).

Carbon clearance assay : Carbon clearance assay was performed according to the procedure of Sljivic [10] with a slight modification. Pelikan fount india drawing ink (518.221143 ; Hannover, Germany) was suspended in phospate-buffered saline (pH 7.5) containing 1% of gelatin, and injected into the tail vein of mice. Blood samples were obtained from the retro-orbital plexus at exactly 4, 7, 10, and 13 minutes after injection of the ink suspension. Each sample was immediately mixed with 0.1% sodium carbonate solution and centrifuged. Optical density of the serum was measured at 660 nm. Clearance rate was calculated by linear regression analysis, and the results were expressed as T1/2.

Histological study : After *in vivo* EIC assay, histological examination was carried out using an established protocol. Liver, spleen, and thymus were excised from each mouse and fixed with 4% formaldehyde. Each specimen was stained with hematoxylin-eosin.

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

Results

Effects of prednisolone on the clearance of immune complexes from the circulation in mice

Twenty days of prednisolone administration resulted in a tendency of liver weight to increase, and produced a significant decrease in the spleen weight as shown in Table I. These observations were agreeable to a number of basic and clinical reports. It has been considered that the liver and spleen have an important role in the immunosystem, and dysfunction of these organs may affect the clearance of immune complexes from the circulation. In the present study, we measured the half-life of clearance of GAG from the circulation. Prednisolone treatment elongated the half-life (T1/2) of GAG significantly in a dose-depending manner.

We therefore used 1.2 mg prednisolone *s.c.* administration in the following experimentation.

Effects of Kampo prescriptions on the clearance of immune complexes of steroid treated mice

The clearance of ICs in C3H/He mice was significantly increased by fourteen days of prednisolone treatment the same as that of ICR mice. When Kampo prescriptions were administered to the prednisolone treated mice through the drinking water, the value of clearance was changed as shown in Table II and Fig. 1. Neither SST nor GRS had any effects on the clearance, but SRT, which was the combination of SST and GRS, shortened T1/2 of the clearance.

Effects of SRT on carbon clearance activity of prednisolone treated mice

T1/2 of carbon clearance of control group was 8.32±0.83 min. (Table III). On the other hand, T1/2 of prednisolone treated group was significantly elongated (10.8±1.48 min, $p<0.01$). When

Table I Effects of prednisolone on body, liver and spleen weight and immune complexes clearance.

	Body weight (g)	Liver weight (g)	Spleen weight (g)	T1/2 (min.)
Control	39.1±2.09	1.810±0.121	0.122±0.016	6.33±0.64
Prednisolone 1.2 mg/kg	37.4±2.73	1.774±0.273	0.100±0.013*	8.12±1.03**
Prednisolone 6.0 mg/kg	38.9±3.16	1.911±0.292	0.077±0.012*	7.92±0.72**
Prednisolone 30 mg/kg	37.7±3.81	1.892±0.281	0.078±0.022**	7.14±0.41*

Each value expresses mean±S.D. (n=7)
Significant difference from the control *$p<0.01$, **$p<0.001$

Table II Effect of Kampo medicines on immune complexes clearance.

	T 1/2 (min.)
Control	7.15±0.37
Prednisolone alone	9.63±1.65
Prednisolone+SST	8.28±1.00
Prednisolone+GRS	8.61±0.57
Prednisolone+SRT	6.55±0.33*

SST ; Sho-saiko-to, GRS ; Gorei-san, SRT ; Sairei-to. Each value expresses mean±S.D. (n=7)
Significant difference from the prednisolone alone *$p<0.05$
The dose of prednisolone 1.2 mg/kg body weight. *s.c.*

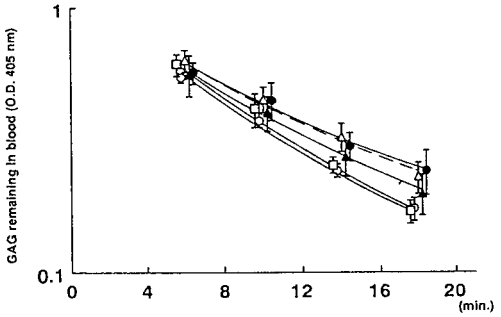

Fig. 1 Effect of Kampo medicines on immune complexes clearance.
○ : Control, ● ; Prednisolone, ▲ : Prednisolone+Sho-saiko-to, △ : Prednisolone+Gorei-san, □ ; Prednisolone+Sairei-to

Table III Effect of Kampo medicines on carbon clearance.

	T 1/2 (min.)
Control	8.32±0.83
Prednisolone alone	10.8±1.48*
Prednisolone+Sairei-to	9.64±0.44*

Each value expresses mean±S.D. (n=7)
Significant difference from the control *$p<0.01$
The dose of prednisolone was 1.2 mg/kg body weight. *s.c.*

SRT was administered to prednisolone treated mice, no significant effect of this Kampo prescription was observed (T1/2=9.64±0.44 min., *vs.* control $p<0.01$).

Histological study

Liver, spleen, and thymus were excised from just after *in vivo* EIC assay, and were examined in a histological study. As shown in Fig. 2, the number of cells in the thymic cortex of the prednisolone treated mouse was obviously decreased, in comparison with the normal mouse (Fig. 2a, b). However, the number of cells was improved by SRT administration (Fig. 2c). Furthermore, cell proliferation was observed around the central artery and marginal zone of white pulp in the spleen from SRT treated mice. No apparent difference was observed in the liver.

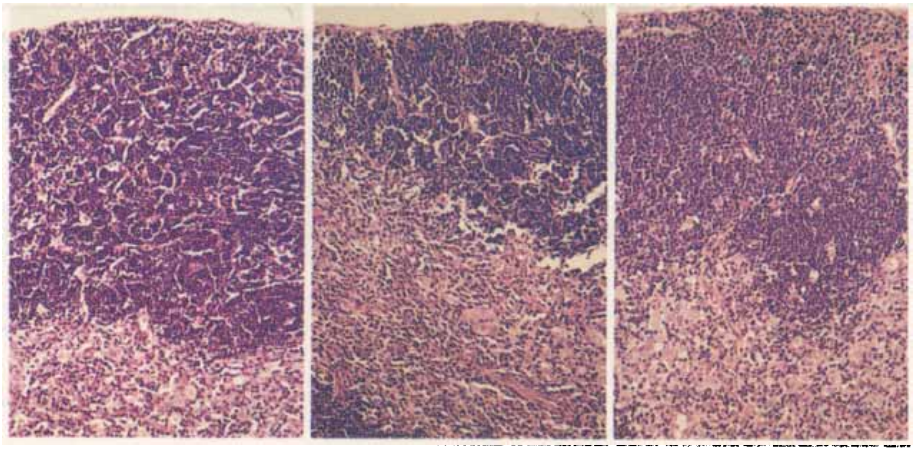

Fig. 2 Effect of Sairei-to on thymus of prednisolone treated mouse.
A ; normal mouse, B ; prednisolone treated mouse, C ; prednisolone and Sairei-to treated mouse. Hematoxylin-eosin stain (×100)

Discussion

Increased ICs formation and/or dysfunction of the mononuclear phagocytic system (MPS) related to ICs clearance may have a role in the pathogenesis of autoimmune diseases.[4,5] Steroids have been generally used for treatment of autoimmune diseases. The effects of steroids, *i.e.* anti-inflammatory effects, suppressing effects on antibody production, and anti-glanulomatous, have been clinically appreciated. However, on the contrary, such drugs have severe adverse reactions and rebound phenomena. Therefore, considerable efforts to find new drugs which improve the immune system have been made at a number of laboratories. In the present study, we tested three Kampo medicines which are clinically used for hepatitis,[11] liver cancer,[12] nephrosis,[13] kidney disorders,[14] and various diseases. There is accumulating evidence which suggest that these Kampo prescriptions have anti-inflammatory effects and anti-allergic effects.[7] We revealed first that prednisolone treatment resulted in a decrease of the spleen weight and an elongation of the clearance time of ICs. This results might be due to dysfunction of MPS, and agree with basic reports.[15,16] Next, we evaluated the effects of three Kampo prescriptions. Neither SST treatment nor GRS treatment represented effects on *in vivo* enzymatic immune complex clearance assay (EIC assay). However, the combination of these two Kampo prescriptions, SRT, treatment represented changes on *in vivo* EIC assay. Elongated T1/2 of ICs clearance (T1/2) of prednisolone treated mice was significantly improved by SRT administration. Also, the improvement was confirmed by the histological study. But the concomitant treatment of SRT and prednisolone slightly increased T1/2 of carbon clearance. These results indicate that SRT might have a potential effect on specific activity of the immune complex through MPS but not on non-specific activity. According to histological study, SRT restored the thymus and the spleen injured by prednisolone treatment. Recently, we reported that GAG bind to macrophages only *via* the cell surface Fc receptors *in vitro*.[17] Therefore, the increase of the specific activity might be due to the enhancement of Fc-receptor dependent clearance for GAG.

The Kampo formulas tested in this experiment were very popular prescriptions. Recently, it was reported that SST regulates immune system, *i.e.* SST enhanced antibody responses,[18] T-cell colony formation, and blastoid transformation.[19] Previously, we also revealed that SST improved the clearance of ICs in NZB/NZW F_1 mice treated with LPS.[8] On the other hand, GRS and SRT have been clinically used for treatment of kidney disorders including one of autoimmune diseases. Abe *et al.*[7] indicated that Sairei-to (another name of Sho-saiko-to-go-gorei-san) increased the activity of the antiglanulomatous action of dexamethasone and enhanced the anti-inflammatory actions of glucocorticoid. In addition, they concluded that Sairei-to had a preventive effects on proteinurea with experimental nephrosis. These observations strongly suggested that these Kampo medicines might be potential treatments for the patients with autoimmune diseases. According to our experiments, SRT may facilitate Fc-receptor dependent clearance, and then enhance the clearance of ICs from the circulation. The action mechanisms of this Kampo medicine on the immune system might be different from that of SST. We also conclude that the appearance of the activity is required that the blend of two Kampo formulas; "Sho-saiko-to" and "Gorei-san". However, both the details of action mechanisms and the active component (s) of these Kampo medicines have been still unclear. Further investigation is needed.

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和文抄録

ステロイド剤投与によって生じる単核食細胞系の機能低下に対し，漢方方剤の影響を検討した。単核食細胞系の機能測定には，*in vivo* enzymatic immune complex clearance assay (EIC assay) および carbon clearance を用いた。*in vivo* EIC assay を用いた検討で，マウスの免疫複合体除去能はプレドニゾロンの投与により，対照群と比較し有意な低下が認められた。プレドニゾロン投与によって生じた免疫複合体除去能の低下に対し，3種の漢方方剤（小柴胡湯，五苓散料および柴苓湯）の影響について検討した結果，柴苓湯投与群において有意な免疫複合体除去能の改善が認められた。一方，carbon clearance の検討では，この改善作用は認められなかった。また組織学的検討で，プレドニゾロン投与によって生じた胸腺皮質の細胞数の減少が，柴苓湯投与により改善された。

以上の結果より，ステロイド剤投与によって生じた単核食細胞系の機能低下に対して，柴苓湯は改善作用を示し，この作用は非特異的な貪食作用の改善によるものではなく，Fc-receptor を介した特異的な免疫複合体除去能の改善によることが示唆された。

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