The Effects of Crude Polysaccharide Fractions of 4 Kinds of Kampo-hozai Administered Orally on Nitric Oxide Production by Murine Peritoneal Macrophages

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In order to characterize the pharmacological role of the crude polysaccharide fraction in kampo-hozai, we chose 4 kinds of kampo-hozai, Shosaiko-to, Daisaiko-to, Hachimi-jio-gan and Hochu-ekki-to, and studied the effects of their crude polysaccharide fractions on nitric oxide (NO) production by 3% thioglycollate-induced murine peritoneal macrophage. Oral administration of these fractions for 7 d augmented lipopolysaccharide (LPS, 0.1–10 µg/ml)-induced NO production by peritoneal macrophages. These results suggest the possibility that a crude polysaccharide fraction affect the macrophage function in most kampo-hozai.

Key words kampo-hozai; crude polysaccharide fraction; macrophage; nitric oxide production

The important role of Japanese and Chinese traditional medicines, so-called kampo-hozai, and their profound influence on the health care system in Japan and China are getting to be well recognized. Many investigators have reported the efficacy of kampo-hozai through various pharmacological and biological studies using *in vivo* and *in vitro* systems. Kampo-hozai are thought to work as balancers of “homeostasis”, the so-called biological response modifier. Shosaiko-to is one of the most widely used kampo-hozai, and it has been widely characterized that it expresses such diverse activity as immunostimulatory, anti-allergic and anti-inflammatory effects. This prescription is clinically used for the treatment of various infectious diseases such as chronic viral hepatitis. Our previous studies revealed that Shosaiko-to augmented the immune response by affecting macrophage functions. The oral administration of Shosaiko-to restored the carrageenan-induced reduction of antibodies to lipopolysaccharide (LPS)¹⁾ and enhanced phagocytosis.²⁾ Furthermore, the oral administration of Shosaiko-to also augmented interleukin 1 release and reduced prostaglandin formation by murine peritoneal macrophages. Shosaiko-to is thought to be a potent macrophage activator. Recently we demonstrated that the immunostimulatory action of Shosaiko-to was due mainly to its crude polysaccharide fraction.³⁾ Since most of kampo-hozai is consisted of plant materials, they should contain polysaccharides. Thus, we chose 3 kinds of prescriptions in addition to Shosaiko-to, and investigated here whether crude polysaccharide fractions of the other kampo-hozai could affect the macrophage function.

MATERIALS AND METHODS

Animals Male ICR mice, 5 weeks old, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The animals were housed in standard plastic cages in an air-conditioned room (24 °C) and a given commercial diet (CE-2, Clea Co., Tokyo, Japan) and water *ad libitum*. The animals were kept for at least 7 d after their arrival.

Chemicals RPMI-1640 and fetal calf serum (FCS) were

purchased from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). LPS (*Escherichia coli* 0111: B4) was from Difco Laboratories (Detroit, MI, U.S.A.). All other chemicals used were of special grade.

Preparation of Crude Polysaccharide Fraction from Each Kampo-hozai Most of the medicinal herbs were authenticated and provided by Tsumura Co., Ltd. (Tokyo, Japan). The mixture of medicinal herbs was extracted with 600 ml of water at 100 °C for 1 h. The decoction was filtered and then lyophilized to obtain a dry extract powder. The powdered extract of each kampo-hozai was dissolved in water and then the addition of 3 volumes of EtOH to this solution gave the precipitate and the EtOH-soluble supernatant. The precipitate was suspended in the appropriate volume of water and then lyophilized to give the powder (EP fraction). The EtOH-soluble supernatant was concentrated and then lyophilized to get the powder (ES fraction).

Chemical Analysis of EP Fractions The total carbohydrate and uronic acid content were determined by the phenol-sulfuric acid⁴⁾ and the sulfuric acid-carbazole methods,⁵⁾ respectively, using glucose and galacturonic acid as the respective standards. Protein was assayed by the Bradford method,⁶⁾ with bovine serum albumin as the standard.

Determination of Nitric Oxide (NO) Production by Murine Peritoneal Macrophages Murine peritoneal macrophages were obtained from ICR mice by lavage with 8.0 ml cold RPMI-1640, 4 d after *i.p.* injection of 2.0 ml of 3.0% thioglycollate broth (Wako Junyaku, Osaka, Japan). Cells were seeded in 96-well cluster plates (Falcon Plastics, NJ, U.S.A.) at a density of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS. After 2 h incubation at 37 °C under 5% CO₂–95% air, nonadherent cells were removed by washing with the medium, and the remaining cells were incubated at 37 °C with the medium containing various concentrations of sample or LPS. After 20 h of incubation, 100 µl of the cell-culture supernatant was added to 100 µl of Griess reagent (0.05% *N*-1-naphthyl-ethylene diamine-dihydrochloride / 0.5% sulfanilamide / 2.5% phosphoric acid) and incubated at room temperature for 10 min in a 96-well microplate. The

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absorbance was read at 577 nm in a Model 450 Microplate Reader (Bio-Rad, CA, U.S.A.). Sodium nitrite was diluted in the medium and used as a standard.

Statistical Analysis Results are given as the mean $\pm$ S.E.M.; mean values are compared by the two-tailed Student's *t* test.

RESULTS

Fractionation of Kampo-hozai In order to fractionate kampo-hozai into high molecular mass fraction and low molecular mass fraction, ethanol precipitation was carried out. Table 1 showed the yields of each kampo-hozai and corresponding fractions. Table 2 showed the results of the chemical analysis of each EP fraction. These data suggested that EP fractions consisted mainly of polysaccharides.

Effects of Crude Polysaccharide Fractions on NO Production by Murine Peritoneal Macrophages *in vivo* In order to investigate the effects of crude polysaccharide

Table 1. Yield of 4 Kinds of Kampo-hozai and Their Fractions

	Kampo-hozai (g)	EP fraction (g)	ES fraction (g)
Shosaiko-to	8.29 $\pm$ 0.04	1.87 $\pm$ 0.01	6.35 $\pm$ 0.11
Daisaiko-to	8.34 $\pm$ 0.02	1.56 $\pm$ 0.04	6.70 $\pm$ 0.07
Hachimi-jio-gan	6.91 $\pm$ 0.07	0.68 $\pm$ 0.05	6.17 $\pm$ 0.01
Hochu-ekki-to	9.01 $\pm$ 0.10	1.60 $\pm$ 0.04	7.25 $\pm$ 0.04

Table 2. Chemical Properties of EP Fractions

EP fraction	Total sugar (as Glc)	Total protein (as BSA)	Total uronic acid (as GalA)
Shosaiko-to	78.0%	2.1%	14.8%
Daisaiko-to	80.0%	2.3%	18.2%
Hachimi-jio-gan	65.4%	4.7%	19.4%
Hochu-ekki-to	75.2%	2.4%	22.2%

Fig. 1. Effects of EP Fractions Administered Orally for 7 d on NO Production by Murine Peritoneal Macrophages

□, control; ■, Shosaiko-to EP; ▨, Daisaiko-to EP; ▩, Hachimi-jio-gan EP; ▤, Hochu-ekki-to EP. Macrophages were prepared from 4 to 5 mice treated with various doses of EP fraction orally for 7 consecutive days. Each column represents the mean $\pm$ S.E.M. of triplicate determinations from a representative experiment that was replicated three times with similar results. ***p* < 0.01 vs. corresponding control group.

fractions on NO production by murine peritoneal macrophages, we obtained thioglycollate-induced macrophages. Crude polysaccharide fractions were administered orally for 7 consecutive days. The dosages of crude polysaccharide fractions are roughly equal to ten times the human dose per day, respectively. As shown in Fig. 1, we did not see NO production in the supernatant cultured without LPS for 20 h. When those macrophages were cultured with 0.1–10 μ g/ml of LPS, NO production was raised at the level of 5.0 ± 1.2 , 18.7 ± 0.6 , 25.5 ± 0.8 μ M in the control group, respectively. Oral administration of crude polysaccharide fractions significantly augmented the NO production. However, the ES fraction did not affect NO production (data were not shown).

DISCUSSION

Macrophages are involved at all stages of the immune response and play a role in the initial response to infection before T- and B-cell-enhanced immunity can act. Mechanisms by which macrophages act as effector cells in a host defense system include both intracellular and extracellular cytotoxic activities. NO has recently been brought into focus as an effector molecule to eliminate microbial invaders and malignancies by murine macrophages.^{7–9} In our previous paper, we reported that Shosaiko-to enhanced phagocytosis and NO production by murine peritoneal macrophages, and this augmentation of macrophage function by Shosaiko-to was due to its crude polysaccharide fraction. Yamaoka *et al.* also reported that the augmentation of NK cells by Shosaiko-to was mainly due to its acidic polysaccharide fraction with a molecular weight of approximately 1.2×10^5 , and that a polysaccharide fraction of Zizyphi Fructus was involved in this activity.^{10–11} Thus, we investigated here whether crude polysaccharide fractions of other kampo-hozai could augment the macrophage function.

As shown in our results, all crude polysaccharide fractions we examined here were able to augment NO production by murine peritoneal macrophages. It has been reported that the oral administration of Hochu-ekki-to induced the resistance against *Listeria monocytogenes* and that macrophage activation was involved in this activity.¹² Thus, the effect of the crude polysaccharide fraction of Hochu-ekki-to could be easily understood. On the other hand, there have been few reports concerning the immunopharmacological actions of Daisaiko-to and Hachimi-jio-gan, although the efficiencies of both prescriptions against hyperlipidemia and experimental diabetes were well investigated.¹³ Both EP fractions, however, showed almost the same effects as the EP fractions of Shosaiko-to and Hochu-ekki-to did. Since most kampo-hozai contain polysaccharides, these results led us to consider a hypothesis that most kampo-hozai could affect the macrophage function. Assuming this hypothesis to be true, we might then partly explain the mechanism of action of kampo-hozai that is believed to work through three systems, such as the central nervous system, the endocrine system and the immune system.

Many polysaccharides from medicinal plants, algae and fungi are known to have various biological activities such

as immunopotentiating, anti-tumor, anti-ulcer and hypoglycemic effects. Tomoda *et al.* reported that some plant polysaccharides showed reticuloendothelial system-potentiating activities in a carbon clearance test *in vivo*, suggesting that these plant polysaccharides could augment the macrophage functions.¹⁴⁻¹⁶ The mechanism of this augmentation on macrophage function by crude polysaccharide fractions is still unclear. In order to study the mechanism of macrophage activation, we should isolate the active compounds from these EP fractions. Thus, further studies on the isolation and chemical characterization of the active principles are now in progress.

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