Effects of Rhei Rhizoma on immune complex clearance *in vitro* I

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Abstract

Circulating immune complexes (CICs) have been shown to be related to the progress of SLE and other, chronic and systemic diseases. A variety of Kampo herbal prescriptions (Japanese traditional medicine) have been reported to possess certain curative effects, and we have investigated those which are reported to produce an enhancing effect in clearing immune complexes. To elucidate the active principle, this investigation was carried out using the peroxidase anti-peroxidase complex (PAP) clearance system with macrophage (Mϕ) (EIC assay). In screening 280 varieties of herbs, 20 were positive in the EIC assay. These herbs accelerated the binding of PAP to Mϕ. Rhei Rhizoma (RR) was shown to have the strongest activity and was the most reproducible. The binding activity of PAP was dose-dependent on the concentration of RR and activity reached a maximum level when Mϕ were incubated with RR for 30 minutes. The amount of PAP binding to Mϕ incubated with RR decreased with time. It is felt that PAP was ingested and digested by Mϕ. Therefore we suggest that CICs are cleared from the blood *in vivo* as will as *in vitro*.

Key words macrophage, EIC assay, Reticuloendothelial system, CICs, Rhei Rhizoma, peroxidase-antiperoxidase complex

Abbreviations BSA, bovine serum albumin ; B/W F_1, NZB/NZW F_1 ; CICs, circulating immune complexes ; CMF-Hanks, Calcium magnesium free Hanks ; EIC assay, enzyme containing immune complex clearance assay ; Mϕ, macrophage ; O.D., optical density ; OPD, *o*-phenylenediamine dihydrochloride ; PAP, peroxidase antiperoxidase complex ; PBS, phosphate buffered-saline ; RR, Rhei Rhizoma

Introduction

Upon exposure to most antigens, an individual responds by producing specific antibodies that may combine with the inciting antigens to form immune complexes. This immune response is designed to eliminate and/or neutralize antigens, and benefiting the host. In some circumstances, however, the process is accompanied by a variety of inflammatory reactions, known collectively as the Type III reaction.[1,2] Circulating immune complexes (CICs) are all responsible for, or associated with, a variety of diseases including neoplastic diseases,[3] hepatitis,[4] asthma,[5] Behcet's disease[6] and many others. Awareness of the important role of CICs in many diseases has encouraged development of a technique for demonstrating their activity in tissues and biological fluids. *In vivo* and *in vitro* experiments have recently elucidated many of the factors involved in CICs' formation, removal, and localization as well as the mechanisms involved in CIC-induced inflammatory reactions.

In spite of advances in the study of immune complex diseases, treatment of patients with these disorders has been performed mainly by the use of glucocorticoid or immunosuppressive agents. There are few therapeutic agents which exert their effect on the elimination of CICs. Since it is well known that CICs in the circulation are cleared through the reticuloendothelial sys-

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tem, it may be assumed that CICs will be effectively removed from the circulation if the function of reticuloendothelial system could be enhanced. There is some evidence suggesting that some Kampo herbal medicines may enhance phagocytic function in the reticuloendothelial system or macrophage population. We therefore have developed an experimental method for assaying immune complex clearance using peroxidase-antiperoxidase complex and applied it to the screening of Kampo herbal medicines.

Materials and Methods

Mice : Six to ten week old male ICR mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan and were used.

Herbs : Two hundred and eighty varieties of plants used in traditional Chinese medicine were screened by the EIC assay. Dried plant material (10 g) was suspended in 300 ml of distilled water and reduced by boiling to 100 ml. In the case of Rhei Rhizoma, 雅黃 (Uchida-Wakanyaku Co., Tokyo), 30 grams of the herb was decocted in 100 ml of water to half of its original volume and then lyophilized.

Peritoneal exudate Mϕ : ICR mice were given intraperitoneally an injection of 2.5 ml of thioglycollate medium (Eiken Chemical Co., Ltd., Tokyo) as an irritant. Ninety-six hours after the injection, Mϕ rich peritoneal exudate cells were obtained by washing the peritoneal cavity with calcium and magnesium free Hanks solution (CMF-Hanks) at pH 7.4 containing heparin, centrifugation at $50 \times g$ for 10 minutes, and recentrifuged twice more in CMF - Hanks solution. Pellets containing peritoneal exudate cells were suspended in RPMI 1640 medium, and the cell number was adjusted to 1×10^6 cells/ml. The cell suspension was cultured in 96 well flat - bottomed polystyrene tissue culture plates (Falcon, Oxnard, CA, USA) in a CO_2 incubator for 60 minutes. Each well contained 0.2×10^6 cells. After incubation, each well was rinsed in CMF-Hanks solution twice to remove the nonadherent cells. Adherent cells were cultured in a 5% CO_2 incubator overnight, and were used in the CICs binding assay.

Peroxidase-antiperoxidase complex (PAP) : PAP (Jackson Immuno-Research Laboratories, Inc., Avondale, Pennsylvania) obtained by immunizing mice was used for CICs. PAP consists of three antigens and two antibodies. It was dissolved in RPMI 1640 containing 1% bovine serum albumin (BSA).

Assay of CICs binding to Mϕ, in vitro enzyme containing immune complex clearance (EIC) assay : Cultured Mϕ from wells were washed with phosphate buffered-saline (PBS). 0.2 ml of water extracts of 280 different plants in RPMI 1640 was then added. After incubation for 30 minutes at 37℃ in 5% CO_2, each well was washed with cold PBS four times. 0.2 ml of PAP (0.2μg/ml) was then added to each well. After incubation for 30 minutes at 4℃, each well was washed again four times with cold PBS. At this time, more than 95% of Mϕ excluded trypan blue. A substrate of peroxidase, *o*-phenylenediamine dihydrochloride (2 mg/ml) (OPD) (Tokyo Chemical Industry Co., Ltd., Tokyo) dissolved in 0.05 M acetate buffer (pH 5.0) with H_2O_2 at a final concentration of 0.05% were added to each well (Mϕ did not burst). After 20 minutes, the optical density at 492 nm was measured with a Titertek Multiskan (Flow Laboratories Inc., McLean, VA). The peroxidase activity indicated the amount of PAP.

Effect of RR as measured by the EIC assay : RR at different concentrations and Mϕ were incubated at 4℃ for 30 minutes. After washing, Mϕ was incubated with PAP at 37℃ for varying time periods. OPD was added, and peroxidase activity was determined. Then, various concentrations of RR and Mϕ were incubated at 4℃ for different time periods. After washing, Mϕ was incubated with PAP at 4℃ for 30 min, and peroxidase activity was measured.

Loss of PAP binding to Mϕ : RR at varying concentrations and Mϕ were incubated at 37℃ for 30 minutes. After washing with PBS, Mϕ was incubated with 0.2 μg/ml of PAP at 4℃ for 30 minutes. Again after washing, RPMI 1640 was added to Mϕ and Mϕ was incubated again for different time periods at room temperature.

After incubation, each well was washed with PBS, and OPD was added. The optical density at 492 nm was determined.

Competition of PAP with other proteins : RR (0.25 mg/ml) and Mϕ were incubated at 37℃ for 30 minutes. After washing, Mϕ and PAP were incubated with serum proteins. After incubation for 30 min at 4℃, each well was washed with PBS and then OPD was added. The optical density at 492 nm was measured.

The effect of tannins on binding of PAP to the surface of Mϕ : Mϕ were incubated with RR, tannic acid (Sigma, St. Louis, MO), (+)-catechin (Sigma), (−)- epicatechin (Sigma), catechol (Tokyo Chemical Industry Co., Ltd., Tokyo), gallic acid (Tokyo Chemical Industry), elaidic acid (Tokyo Chemical Industry) and pyrogallic acid (Wako Pure Chemical Industries Ltd., Tokyo). After incubation for 30 min at 37℃ in 5% CO_2, each well was washed and 0.2 ml of PAP (0.2 μg/ml) was added. After incubation for 30 min at 4℃, each well was washed and OPD was added. The optical density at 492 nm was determined.

Results

Screening

Water extracts of 280 different plants were tested by the EIC assay. Strong activity was observed in Polygonaceae, Zingiberaceae and Ranunculaceae (in preparation). Of these, Rhei Rhizoma was shown to be the most intense. Its activity was also the most reproducible.

The effect of RR on the EIC assay

RR at various concentrations and Mϕ were incubated at 37℃ for 30 minutes. Each well was washed with PBS. The binding of PAP to Mϕ was determined. As shown in Fig. 1, there was little change in the binding of PAP to Mϕ when Mϕ was incubated with PAP for more than 10 minutes. The optical density was maximal at an RR concentration of 0.5 mg/ml and an incubation time of 120 min. The relationship between the concentration of RR and PAP bound to the surface of Mϕ was dose dependent.

RR at different concentrations and Mϕ were incubated at 37℃. Each well was washed with cold PBS. The binding of PAP to Mϕ was determined. As shown in Fig. 2, the optical density was maximal after incubating with 0.5 mg/ml of RR and Mϕ for 30 minutes. However the optical density decreased or remained constant after an incubation time of more than 30 minutes at all concentrations of RR tried.

Loss of PAP binding to Mϕ

After PAP was bound to the surface of Mϕ,

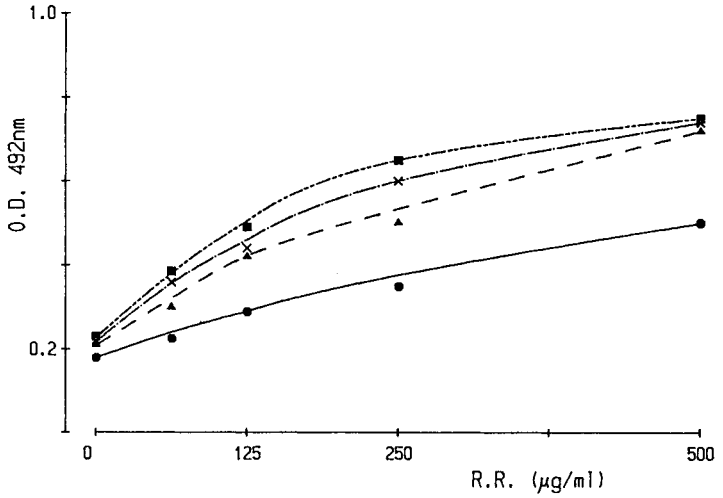

Fig. 1 Time course of the binding of PAP to Mϕ.
Mϕ and RR were incubated at 37℃ for 30 min. After washing, Mϕ was incubated with PAP at 4℃ for 10 (●—●), 30 (▲···▲), 60 (×–·–×), 120 (■···■) minutes, and the activity of peroxidase was measured.

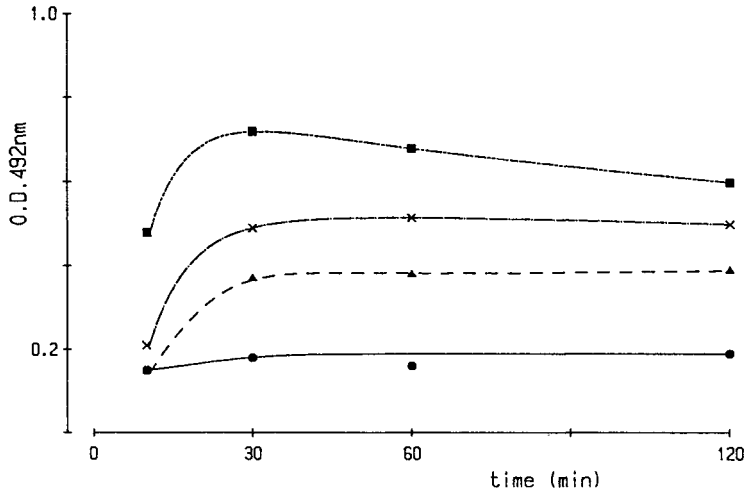

Fig. 2 Effect of Rhei Rhizoma on the binding of PAP to Mϕ.
Mϕ and RR (0 μg/ml : ● — ●, 125 μg/ml : ▲ ··· ▲, 250 μg/ml : ×-·-×, 500 μg/ml : ■-·-■) were incubated at 37℃ for various time. After washing, Mϕ was incubated with PAP at 4℃ for 30 minutes, and the activity of peroxidase was measured.

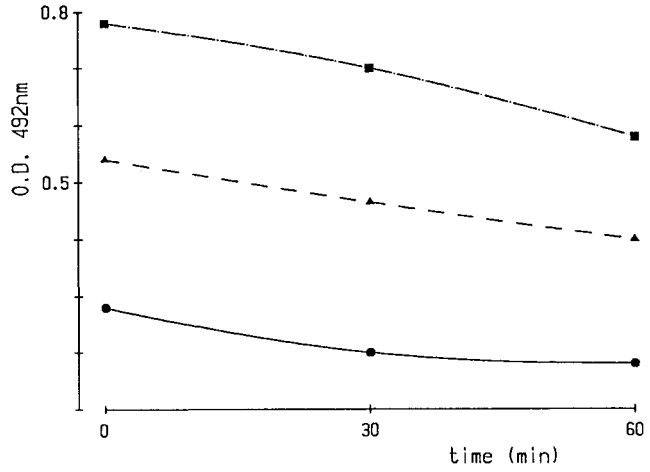

Fig. 3 Effect of the ingestion of PAP by Mϕ.
Mϕ and RR (0 μg/ml : ● — ●, 250 μg/ml : ▲ ··· ▲, 500 μg/ml : ■-·-■) were incubated at 37℃ for 30 minutes. After washing, Mϕ was incubated with PAP at 4℃ for 30 minutes. After washing, RPMI 1640 was added to Mϕ and ingestion and dissociation were measured.

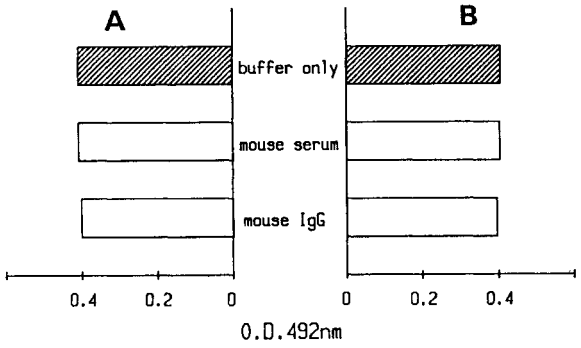

Fig. 4 Effect of mouse serum and mouse IgG on the binding of PAP to Mϕ.
Mϕ and RR were incubated at 37℃ for 30 minutes. After washing, Mϕ and PAP were incubated with 2 μg/ml (A) or 2 mg/ml (B) of mouse serum or mouse IgG at 4℃ for 30 minutes.

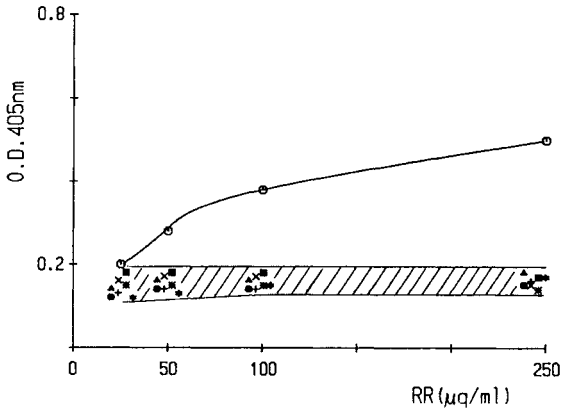

Fig. 5 Effect of various tannins on the binding of to Mϕ.
Mϕ were incubated with RR (○—○), tannic acid (●), catechin (▲), epicatechin (■), catechol (×), gallic acid (☆), elaidic acid (+) and pyrogallic acid (*) at 37℃ for 30 minutes. After washing, Mϕ was incubated with PAP at 4℃ for 30 minutes and the activity of peroxidase was measured.

RPMI 1640 was added, and the mixture incubated again fos different time periods at room temperature. After incubation, each well was washed, and OPD was added. The optical density was determined. As shown in Fig. 3, at any concentration of RR, the amount of PAP binding to Mϕ decreased with time, reaching a maximum at 0.5 mg/ml of RR. PAP binding to Mϕ was thought to be ingested or dissociated.

The effect of other proteins on binding of PAP to Mϕ

PAP was added to Mϕ and incubated in 0.25 mg/ml of RR. Then Mϕ and PAP were incubated with buffer, mouse serum or mouse IgG. As shown in Fig. 4, other proteins (2 μg/ml or 2 mg/ml) did not inhibit the binding of PAP to Mϕ, and mouse IgG did not inhibit PAP binding to Mϕ.

The effect of tannins on binding of PAP to the surface of Mϕ

In order to determine whether the binding of PAP to Mϕ was nonspecific, Mϕ was incubated with RR, tannic acid, (+)-catechin, (−)-epicate-

chin, catechol, gallic acid, elaidic acid and pyrogallic acid. As shown in Fig. 5, the tannins had no effect on the binding of PAP to the surface of Mϕ, however RR had a very significant effect.

Discussion

Of the 280 kinds of herbs studied, 20 were shown to be a positive in the EIC assay. Most of these herbs contain tannin, which has protein-denaturing properties. Of the 20 positives Rhei Rhizoma (RR) was shown to have the strongest and most reproducible activity.

RR was originally described in the Chinese book *Shen Nung Pên Ts'ao Ching* from the Han Dynasty, which states that RR has approximately ten pharmacological properties. RR was introduced to many other countries from China and had generally been used as a purgative. It was recently shown that RR has several pharmacological properties apart from its purgative action.[9-11,21] We have studied one of these properties by the EIC assay.

PAP,[12] used as CICs, consists of mouse anti-horse radish peroxidase and horse radish peroxidase. The detection of PAP replaces the measurement of peroxidase activity. It is convenient that the antibody and Mϕ are from the same species. CICs in the blood are cleared by the reticuloendothelial system, especially by mononuclear phagocytes.[7,13] CICs bind to the surface membranes of the cells via Fc receptors and are then subsequently are phagocytosed and ingested.[14,15] It is reported that the majority of CICs are cleared by the liver via the Fc receptors, but that this bears no relationship to the C3 receptor.[16,17] Though PAP is not bound to complement, this assay system can be used as a clearance model of CICs *in vitro*.

The binding ability of PAP to Mϕ was dose-dependent on the concentration of RR, but this ability increased only slightly when Mϕ were incubated with RR for more than 30 minutes. This may be due to the toxicity of RR. Incubating Mϕ with PAP for more than 30 minutes might have little effect on the binding of PAP to Mϕ, because the Fc receptor of the Mϕ was saturated. The amount of PAP binding to Mϕ decreases during incubation (Fig. 3). One explanation for this could be that the PAP were endocytosed and ingested by Mϕ,[18] or that they were separated from Mϕ. The dissociation of CICs after binding to Mϕ is insignificant within 60 minutes.[14,19] Therefore, the level of CICs in the blood was expected to decrease, partially through endocytosis and digestion by Mϕ, and partially through transport to the liver. When B/W F_1 mice were administered with non-purgative RR, the value of CICs[8] in the blood tended to decrease more than that in the control group (Data not shown). It is reported that the CICs clearance system in B/W F_1 mice is disordered or overloaded by pathogenic immune complexes,[20] but in this study it was shown that RR improved this condition, and that the value of CICs decreased. This supports results of the *in vitro* experiments.

There was no effect on the binding of PAP to the surface of Mϕ when competing with mouse serum and mouse IgG. It is thought that mouse IgG dose not inhibit this binding because it easily dissociated from the Mϕ. Therefore we believe that this binding is specific, and that it may be effected through the Fc receptor. It is expected that CICs are cleared specifically via the Fc receptor *in vivo*.

It is reported that the tannin-rich fraction in RR specifically enhances the response with the receptor.[21] The capability of CICs in binding to Mϕ is thought to depend on the fraction used and will be the subject of future research.

When studying the pharmacological effect of plants, the denaturing property of tannins on a protein must be considered. Several varieties of tannin, *e.g.* (−)epicatechin, catechol and gallic acid, had no effect on the ability of CICs to bind to Mϕ. It is felt thefore that the binding of PAP to Mϕ was not through the nonspecific binding effects of tannin.

It was shown that RR enhances the ability of reticuloendothelial system to clear CICs, and that RR may actually decrease the level of CICs in the blood. Consequently it is possibe that RR can be employed in a new type of treatment of immune complex diseases. *In vitro* studies of CICs may

be made possible through this CICs clearance assay system.

和文抄録

循環免疫複合体（CICs）がSLEなど，いわゆる難病の活動性と関連していることが注目されている。和漢生薬を用いた治療によりこれらの疾患が改善する例が報告されていることから，生薬のCICsクリアランスへの影響について検討を行った。CICsとしてperoxidase-antiperoxidase complex（PAP）を用い、マクロファージ（Mφ）によるそのクリアランスを検討した（EIC assay）。280種の生薬についてスクリーニングを行ったところ，20種の生薬がEIC assayで陽性を示した。すなわちこれらの生薬はMφとPAPの結合を促進した。これらのうち大黄の活性が最も強く再現性があった。大黄の活性は，用量依存的で，Mφと大黄とのincubationの時間が30分で最大に達した。Mφと結合したPAPは時間とともに減じた。これはPAPがMφに取り込まれ，消化されていることを示す。したがって，CICsは *in vivo* でも同様に血中から除去されることが期待された。

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