

## A New Pharmacological Testing Method—Different Effects of Levamisole and the Serum of Mice Orally Treated with Levamisole on Mitogenic Activity of Lipopolysaccharide—

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**The direct addition of levamisole to murine splenic lymphocytes had no effect on the mitogenic activity of lipopolysaccharide. However, the addition of serum of mice orally treated with levamisole increased the mitogenic activity, and this increased activity using serum was similar to the result obtained in an *in vivo* experiment. These results suggest that the new *in vitro* experimental method using serum may be able to reproduce the *in vivo* effect of drugs.**

**Keywords** screening method using serum; levamisole; mitogenic activity; lipopolysaccharide; murine lymphocyte

Experiment *in vivo* are of limited use in the study of a drug's mechanism of action, and such investigations inevitably require some *in vitro* work. Namely, the drug under investigation is usually added directly to the medium of cells or exposed to enzymes. However, in many cases the results obtained from the *in vitro* investigation are different from those obtained *in vivo*, and the clinically useful effects of drugs may not be apparent in an *in vitro* experimental system. Therefore, we have been studying the actions of serum from animals orally treated with medicines with the aim of establishing a new *in vitro* experimental system with certain of the characteristics of an *in vivo* system. In our previous studies,<sup>1,2)</sup> we reported the actions of sera obtained from mice orally treated with kampochozai (Japanese and Chinese traditional medicine) and some herbal drugs whose action mechanisms are unknown. When serum obtained from mice orally treated with Shosaikoto, one of Kampochozai, which is reported to be an immunomodulator<sup>3)</sup> was added to cultured spleen cells, no mitogenic action was apparent. On the other hand, the mitogenic action was obtained by the direct addition of extracts of Shosaikoto to the cultured cells. Furthermore, the inhibitory effects on cyclooxygenase activity of the serum obtained from mice orally treated with herbal drugs were also different from those of the herbal drugs extracts added directly. These data suggest that the result obtained from the serum-addition experiment will be different from that obtained from the conventional *in vitro* experiment when the mixture contains an active component which is not absorbed from the intestine, when the mixture contains an inactive component which is transformed to an active one by intestinal flora or by the liver enzymes, or when the mixture contains a component which stimulates a second messenger to show indirect action. Furthermore, we expected that these experimental problems would also arise in the study of western medicines. Therefore, we examined prednisolone and indomethacin in the same experimental systems. When serum obtained from mice orally treated with prednisolone was added to cultured spleen cells, the mitogenic action of lipopolysaccharide was decreased in a dose-dependent manner. This decrease of mitogenic action paralleled that of directly added prednisolone. But, the action of serum was stronger than that of directly added prednisolone by 20%. When the serum of mice treated with indomethacin was added to the bovine seminal microsomal fraction, it inhibited cyclooxygenase activity, and its effect was stronger than that of directly added indomethacin by

30%. These results suggest that the sera contain active metabolites of prednisolone and indomethacin, respectively. Even when a medicine is transformed to active metabolites in the digestive tract or liver, the addition of serum would be advantageous to predict the clinical effect.

In this paper, we report the difference of the pharmacological action of levamisole in the two experimental systems and discuss the desirability of using serum in an *in vitro* system.

### Experimental

**Animals** C57BL/6 mice, 8 weeks old, were purchased from Charles River Japan Inc. (Atsugi, Japan). They were kept in an air-conditioned room (24°C) and given commercial diet, CE-2 (Clea, Tokyo, Japan), and water *ad libitum*.

**Reagents** Lipopolysaccharide (LPS) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Levamisole was obtained from Aldrich Chemical Co., Inc. (Milwaukee, U.S.A.). RPMI 1640 medium was purchased from the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan). Fetal calf serum (FCS) was obtained from Nippon Bio-supp. Center (Tokyo, Japan). Other chemicals were of analytical grade.

**Experimental Procedure** Mice were calmed by daily handling once a day in the morning for 7 d to avoid stress. Mice were decapitated at 10:00—12:00. Levamisole, 3, 10 or 30 mg/kg, dissolved in 0.2 ml of saline was administered 60 min before the decapitation. Blood was collected and allowed to stand for 1 h at room temperature, then centrifuged at  $1000 \times g$  for 10 min. Serum obtained was lyophilized and stored at  $-20^\circ\text{C}$ . Powdered serum was dissolved in the RPMI 1640 medium by adjusting the volume to the original serum volume, and a 10  $\mu\text{l}$  aliquot was added to the 1-ml incubation mixture. Levamisole dissolved in RPMI 1640 solution was also added to the 1-ml incubation mixture.

**Determination of Levamisole in the Serum** A mixture of 3 ml of water, 0.5 ml of 0.1 N NaOH and 4,4'-diaminophenylmethane as an internal standard was added to 0.2 ml of serum. Then the mixture was extracted with  $\text{CHCl}_3$  and the organic layer was evaporated *in vacuo*. The residue was dissolved in 20  $\mu\text{l}$  of MeOH and 10  $\mu\text{l}$  was injected into a high performance liquid chromatography (HPLC) column. The conditions of HPLC were as follows; Shimadzu LC-6A equipped with an SPD-6A UV-VIS spectrometric detector; column, YMC-ODS (150  $\times$  4.6 mm i.d.); detection, 225 nm; eluent, mixed solution of  $\text{H}_2\text{O}$ -MeOH-AcOH (81:19:0.2) containing 10 mM heptanesulfonic acid.

**Preparation of Spleen Cell Suspensions** After the removal of spleen from mice, a cell suspension in RPMI 1640 medium was prepared by repeated gentle pipetting. The product was then passed through a 200-gauge stainless steel sieve and centrifuged at  $600 \times g$  for 10 min. The packed cells were washed three times with RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 5% FCS.

**Mitogenicity** Spleen cells were cultured in culture plates (SH-T24FSW, Terumo Co., Ltd., Tokyo, Japan) at a cell density of  $1 \times 10^6$  cells/ml in a total volume of 1 ml. Culture plates were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air.<sup>4)</sup> LPS, 20  $\mu\text{g}/\text{ml}$ , was added to the culture after the addition of 10  $\mu\text{l}$  serum or drug solution. Tritiated thymidine, 0.5  $\mu\text{Ci}$ , was added to the culture at 24 h. Cells were collected at

48 h on glass fiber filters and the incorporation of tritiated thymidine into the cells was determined with a liquid scintillation counter. Experiments were repeated 6 times using different splenic cell preparations, and the results were expressed as mean cpm  $\pm$  S.E.M. of 6 experiments. Group comparison was performed by the use of Student's *t*-test.

## Results and Discussion

Figure 1 shows that the concentration of levamisole in serum increased in proportion to the oral dose. Then, serum (10  $\mu$ l/well) and levamisole at a dose corresponding to its concentration in serum were added to cultures of spleen cells which had been obtained from mice. Figure 2 (A) shows the effect of serum on the mitogenic activity of LPS. The sera obtained from mice treated with 10 and 30 mg/kg of levamisole increased the mitogenic activity of LPS. These sera contained 1.1 ng (per 10  $\mu$ l of serum) and 3.5 ng (per 10  $\mu$ l of serum) of levamisole, respectively. Therefore, corresponding amounts of levamisole were directly added to the cultured cells and the results were compared with those obtained using serum. As shown in Fig. 2 (B), direct addition of levamisole at doses of 0.1 and 1 ng/well had no effect on the mitogenic activity of LPS, though a dose of

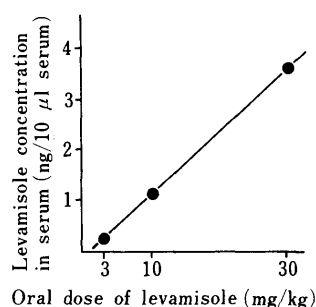


Fig. 1. Levamisole Concentration in Serum after the Oral Administration

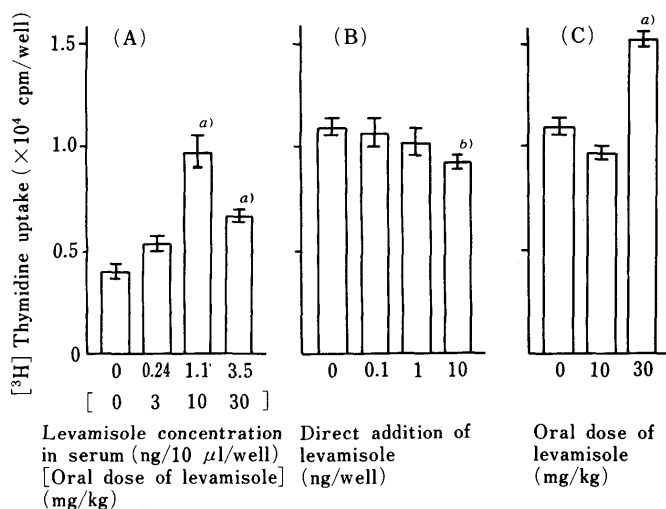


Fig. 2. Effects of Levamisole on the Mitogenic Activity of Lipopolysaccharide

(A) The sera (10  $\mu$ l) obtained from mice pretreated with levamisole (3, 10 or 30 mg/kg) were added to the culture of splenic cell preparation isolated from non-treated mice. (B) Levamisole contained in 10  $\mu$ l of serum (A) was added to the culture of the same splenic cell preparation isolated from non-treated mice. (C) The responses of the splenic cells isolated from mice pretreated with levamisole (3, 10 and 30 mg/kg) for 1 week were measured. The splenic cells at a density of  $1 \times 10^6$  cells/well (1 ml) were incubated for 48 h at 37°C in a 5% CO<sub>2</sub> incubator. Twenty four hour before harvesting, 0.5  $\mu$ Ci of tritiated thymidine was added to the culture. Vertical bars indicate the standard error (S.E.) of 6 samples. *a)*  $p < 0.01$ , *b)*  $p < 0.05$  vs. each control group.

10 ng/well suppressed it. LPS is the B cell mitogen and does not stimulate T cell function *in vitro*.<sup>5)</sup> LPS, moreover, stimulates monocyte or macrophage functions. On the other hand, levamisole is reported to activate T lymphocyte and macrophage function<sup>6,7)</sup> and not to activate B lymphocyte. Our data indicate that the contamination with monocytes or macrophages in the spleen cell culture is negligible because of the ineffectiveness of the direct addition of levamisole. These reports explain that the direct addition of levamisole is inactive on the action of B cell mitogen. Increasing activity of serum obtained from mice pretreated with levamisole on the mitogenic action of LPS may be due to the existence of active substances secreted from T lymphocytes or macrophages, which were stimulated by the oral administration of levamisole. Furthermore, the serum may contain some active metabolite which stimulates B cell function. Mice were then orally given either 10 or 30 mg/kg of levamisole daily for 1 week and the responses of their spleen cells to LPS were compared with those of spleen cells obtained from water-treated mice to evaluate the *in vivo* action of levamisole. As shown in Fig. 2 (C), the spleen cells of mice receiving 30 mg/kg of levamisole increased the mitogenic activity of LPS. The good agreement between Fig. 2 (A) and Fig. 2 (C) shows that the experimental system using serum obtained following drug administration may accurately reflect the *in vivo* results. Although the difference of the administration period is difficult to take account of in evaluating the comparability of the two experimental systems, the stimulation of immune responses by both single and consecutive administrations of levamisole as reported by other investigators<sup>8-10)</sup> may mean that the differences of the administration period are not critical. The peak of levamisole in blood after oral administration is reported to occur at 1 or 2 h, and its half-life is 4 h.<sup>11,12)</sup> In our experiment, the serum obtained 30, 60 and 120 min after the administration showed similar effects on the mitogenic action of LPS. These data show that the appropriate time of blood collection for the determination of levamisole is 1 or 2 h. As regards the metabolism of levamisole, an active metabolite is reported to appear at 24–48 h.<sup>13)</sup> However, the metabolite can not be used as a standard compound for the evaluation of serum obtained, since its structure is unknown. This is also the reason for the collection of blood at 1 h, so that levamisole itself is the appropriate standard compound for the evaluation of serum used. Detailed information on the metabolism of levamisole will be needed to evaluate other times of blood collection. In this experiment, the phenomenon that the mitogenic action of LPS in the serum-treated control group was lower than that in the serum-nontreated control group suggests the presence of some inhibitors in serum. This further indicates that the action of levamisole may be explained in terms of a blocking characteristic against the serum component that inhibits the mitogenic action of LPS. Ramot *et al.*<sup>14)</sup> reported the possibility that levamisole eliminated a blocking factor against T cell function on the basis of a serum transfer experiment. In pharmacological tests on poorly understood medicines, structural changes of the drug by gastric juice, intestinal flora and metabolizing enzymes in liver may change the action of drug. Our previous paper reported that saikosaponin a and saikosaponin d, isolated from *Bupleurum*

*falcatum* L and possessing noteworthy pharmacological effects,<sup>15-18)</sup> were transformed to 15 metabolites in the alimentary tract and those were absorbed into the bloodstream.<sup>19,20)</sup> The actions of these metabolites are likely to be very important and should not be neglected. Furthermore, the serum obtained following the oral administration of a drug may allow the evaluation of indirect drug actions mediated by endogenous substances such as cytokines, hormones and lipids. The possibility of the participation of serum factors was reported in the immunological action of levamisole<sup>21)</sup> and in the antitumor effects of len-tinan<sup>22)</sup> and picibanil.<sup>23-25)</sup> These factors also should be studied precisely to establish the action mechanisms of medicines. This new method of drug evaluation should be useful to predict the clinical effects of drugs by an ordinary *in vitro* testing method and may provide important insights in the cases of drugs whose metabolism is unknown and complex preparations such as traditional herbal medicines.

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