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Addition of *Maitake* D-fraction Reduces the Effective Dosage of Vancomycin for the Treatment of *Listeria*-Infected Mice

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ABSTRACT—*Maitake* D-fraction, β1,6-glucan having β1,3-branches, has been reported to activate the immune system of the host. To elucidate whether the D-fraction can reduce the clinical effective dosage of antibiotics in the treatment of opportunistic bacterial infection, we examined the effects of D-fraction on the treatment of *Listeria monocytogenes*-infected mice in combination with vancomycine (VCM), the only antibiotic used for methicillin-resistant *Staphylococcus aureus* (MRSA). *Listeria*-infection was introduced by its inoculation into the abdominal cavity of mice. Without treatment, all inoculated mice died within 3 days after the inoculation. In contrast, in the mice treated with combined therapy of D-faction (10 mg/kg per day) and VCM (10 mg/kg per day), the survival rate was maintained at 60% on the 10th day after the inoculation, which was superior to that of mice treated with VCM alone (10 mg/kg per day). To investigate the mechanism underlying the reinforcement of VCM treatment by the D-fraction, the activities of macrophages and splenic T cells of *Listeria*-infected mice were evaluated. In mice administered with both D-fraction and VCM, macrophages produced 2.7 times as much interleukin-1 as that of non-treated control mice. The bactericidal activity of splenic T cells was also enhanced by 2.6 times of that of non-treated control mice. These results indicate that D-fraction activates immuno-competent cells, induced cytokine production, and consequently enhanced the bactericidal activities of the splenic T cells against *Listeria monocytogenes*, suggesting the clinical benefit of D-fraction in the case of anti-bacterial treatment for patients with high risks.

Keywords: *Maitake* polysaccharide, *Listeria monocytogenes*-infection, Bactericidal activity, Vancomycin

The gram-positive bacterium *Listeria monocytogenes* (*L. monocytogenes*), cause of the listeriosis, are food-mediated bacteria. This bacteria is an intracellular pathogen and able to grow rapidly in macrophages (1). Therefore, concominant intracellular killing and survival of *L. monocytogenes* occurs in the same macrophages, explaining why the bacteria can escape bactericidal mechanisms in the host defense system. *L. monocytogenes* infection usually occurs in a so-called high risk group, including new neonate, aged people, and patients with cancer, prolonged administration of steroid and compromised immune system (2, 3). As well as *L. monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) easily infects the high risk group individuals. Simultaneous infection with both bacteria commonly occurs in these patients. For the treatment of such a case, vancomycin (VCM) is usually chosen first because this is the only effective antibiotic for MRSA and is also effective against *L. monocytogenes*. However, it is known that a high dosage of VCM causes various side effects, including renal disfunction and hearing disturbance. Considering the general condition of bacteria-infected patients with high risk, treatment with minimun dosage of mono-antibiotic, not of multi-antibiotics, is recommended.

We have previously reported that the D-fraction (β1,6-glucan having β1,3-branches), a component of the fruit body of *maitake* (4, 5), is one of the BRM (biological response modifier), like lentinan (6) which exists in the *Letinus edodes*. These polysaccharides stimulate immune systems by activating immuno-competent cells and introduce an anti-tumor effect. It was found that the D-fraction not only directly activates various effector cells such as macrophages, natural killer cells and cytotoxic T cells that attack tumor cells, but also potentiates the releases of various mediators including lymphokines and interleukin (IL)-1 (4). Also, we have already tried a non-randomized clinical study of the D-fraction therapy for patients of lung,

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breast and liver cancer, in combination with anti-cancer drugs (7). In improved patients to whom the combined therapy was applied, immuno-competent cells, macrophages and T cells, were activated and IL-2 production was enhanced. In addition, we found that combined treatment with the D-fraction and mitomycin C potentiated the tumor growth in mice, suggesting that the D-fraction can effectively reduce the minimum dosage of mitomycin C, required for its anti-tumor action (unpublished data). Furthermore, in our preliminary clinical study, addition of the D-fraction improved a patient who suffered from both MRSA and *Listeria*-infections without changing the dosage of VCM (unpublished data). These findings lead to the idea that immunotherapy using the D-fraction in combination with antibiotics restores the damaged immune and bactericidal functions of high-risk group patients and reduced the dosage of VCM, effective for not only MRSA, but also secondary infection including Lysteriosis. To prove this, we examined the effects of *maitake* D-fraction on the treatment of *Listeria*-infected mice in combination with VCM.

MATERIALS AND METHODS

Materials

Dried powder made from the fruit body of *maitake* (Yukiguni Maitake Co., Niigata) was used. VCM hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Rosewell Park Memorial Institute (RPMI)-1640 medium, phosphate-buffered saline (–) (PBS (–)) and nutrient medium were obtained from Nissui Seiyaku Co. (Tokyo). Mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kit was purchased from Genzyme Co. (Minneapolis, MN, USA).

Animals

Male JcI:ICR mice (5-week-old) were provided from Japan Clea Co. (Tokyo). These mice were given food and water ad libitum until used.

Bacteria

The *L. monocytogenes* were contributed by Kobe Public Health Institute and were passaged through ICR male mice to keep their pathogenic activities. These bacteria were suspended in PBS (–) at the concentration of 1×10^9 CFU (colonies forming unit)/ml.

Preparation of D-fraction

D-fraction of *maitake* was prepared according to the scheme shown in Fig. 1. The D-fraction, which is a glycoprotein, 90% of which is sugar and 10% protein, was purified by DEAE-cellulofine (Biochemical Industries Co., Tokyo). The anthrone method (8) was used to determine polysaccharide.

Preparation of L. monocytogenes-infected mice, administration of drugs and evaluation of survival rate

To prepare *L. monocytogenes*-infected mice, the mice (6-week-old) were inoculated with each of 0.1 ml (1×10^9 CFU/ml) of *L. monocytogenes* into their abdominal cavities. For the treatment with VCM alone, these mice were intraperitoneally (i.p.) administered with various concentrations of VCM (5, 10, 15, 20, 30 mg/kg per day) 24 h after the inoculation. The percentage of surviving mice to the total 20 mice inoculated with *L. monocytogenes* at the start of experiment was evaluated as the survival rate. The experiment was continued for 10 days.

For the treatment with D-fraction and VCM together,

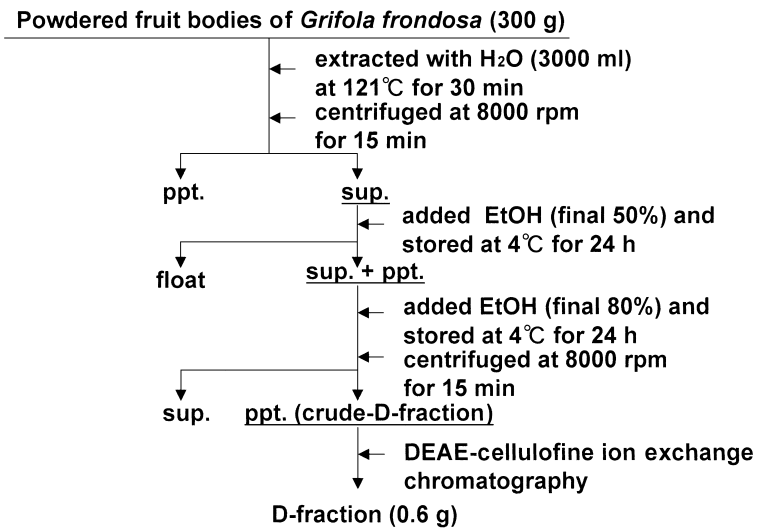

Fig. 1. Preparation of D-fraction from *maitake* (*Grifola frondosa*).

the mice (6-week-old) were injected i. p. with 0.1 ml of 0.9% NaCl or D-fraction (10 mg/kg per day) on the 3rd, 6th and 9th day. On the 10th day, 0.1 ml (1×10^9 CFU/ml) of *L. monocytogenes* was inoculated into their abdominal cavities. Twenty-four hour later, a) 0.1 ml of 0.9% NaCl (in D-treated group) or b) 10 mg/kg per day of VCM (in VCM+D-treated group) was administered i.p. to the mice treated with D-fraction beforehand. Simultaneously, c) 20 mg/kg per day of VCM (in VCM-treated group) or d) 0.1 ml of 0.9% NaCl (in non-treated control group) were injected to the mice already treated with 0.9% NaCl. Survival rate was taken from each group independently.

Measurement of living L. monocytogenes in abdominal cavity

L. monocytogenes-infected mice were killed by cervical dislocation 2 days after their inoculation. The abdominal cavity was opened by dissection with scissors and was repeatedly washed with PBS (–). The PBS wash containing various peritoneal cells, such as macrophages, lymphocytes, bacteria, etc. was collected for later analysis for counting the number of living *L. monocytogenes*. Briefly, the liquid prepared from the abdominal cavity was diluted with PBS (–) and was spread on nutrient agar medium containing 0.4% glucose and cultured at 37°C for 24 h. The CFU was estimated from the number of colonies formed on the medium.

IL-1β production by peritoneal macrophages

Peritoneal macrophages were prepared as described previously (9). PBS washes from the mice abdominal cavity, prepared as described above, were centrifuged at 1000 × rpm (05PR-22; Hitachi, Tokyo) at 4°C for 10 min. The precipitated cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS) (8×10^5 cells/ml) and then cultured at 37°C in 5% CO_2 atmosphere for 2 h. After removing the supernatant with non-adherent cells, adherent cells, mostly peritoneal macrophages, were scraped and washed with 37°C PBS (–), mixed with 5 ml of 12.5 mM EDTA in cold PBS (–) and cooled with ice for 20 min. Macrophages collected by centrifugation (1000 × rpm at 4°C for 10 min) were suspended in RPMI-1640 medium containing 10% of FCS, spread onto 96 well plates (5×10^4 cells/well) and cultured at 37°C in a 5% CO_2 atmosphere for 72 h. After that, the culture medium was centrifuged at 1350 rpm at 4°C for 15 min. Supernatants were collected and assayed for IL-1β with the ELISA method.

Preparation of splenic T cells

A *L. monocytogenes*-infected mouse was killed by cervical dislocation 6 days after the *L. monocytogenes* inoculation. Mouse spleens were taken out and washed with PBS (–). First, the whole spleen cells were prepared according to the modified method described previously (9). The mice spleens were passed through nylon mesh (ϕ150 μm), washed with PBS (–), and centrifuged at 1200 rpm for 5 min. Five milliliters of hemolytic buffer was added to the precipitates to remove erythrocytes. After another centrifugation, the precipitate was suspended with 2 ml of RPMI-1640 medium containing 5% FCS, then layered evenly with Ficoll whose specific gravity was 1.096, and centrifuged at 2000 rpm at 20°C for 20 min. The middle layer was collected and mixed with RPMI-1640 medium and centrifuged again at 1200 rpm for 5 min. The last precipitation was suspended in RPMI-1640 medium containing 5% FCS. Splenic T cells were separated from the whole mouse spleen cells by using the nylon fiber column T (Wako Pure Chemical Industries Ltd., Osaka) supplied as a column for T cell separation.

Bactericidal activity of splenic T cells

Bactericidal activities of T cells were examined according to the method described by Joag and Young (10). Separated splenic T cells were put onto 96 well plates by 1×10^6 cells /well. *L. monocytogenes* was added to those well plates by the amount of 2×10^4 CFU/well so that the effector/target (E/T) ratio becomes 50. Cells were cultured in RPMI-1640 medium containing 5% FCS at 37°C in 5% CO_2 atmosphere for 18 h. After centrifuging them at 1350 rpm at 4°C for 10 min, the supernatant (100 μl) was diluted with PBS (–) by 10000 times, 100 μl of which was put onto nutrient agar medium containing 0.4% glucose, cultured at 37°C for 24 h, and then formed colonies were counted and evaluated as bactericidal activity of splenic T cells.

Statistical analyses

Statistical significance of survival rate were analyzed by the x^2-test. Statistical significance of other data was analyzed by Student's *t*-test. *P* value of less than 0.05, 0.01 or 0.001 was considered as indicating statistical significance.

RESULTS

Survival rate of L. monocytogenes-infected mice under the treatment with VCM alone

Figure 2 shows the dose-dependent survival effects of VCM on *Listeria*-infected mice. The investigation was made between the dosage of 5 mg/kg per day and 30 mg /kg per day. The mice did not survive more than 3 days after the bacteria inoculation, when VCM dosage was 5 mg/kg per day. With 30 mg/kg per day of VCM, the survival rate was the highest. More than 50% survival rate was achieved with the VCM dosage of 20 mg/kg per day, and it was unchanged from the 5th day to the 10th day. The

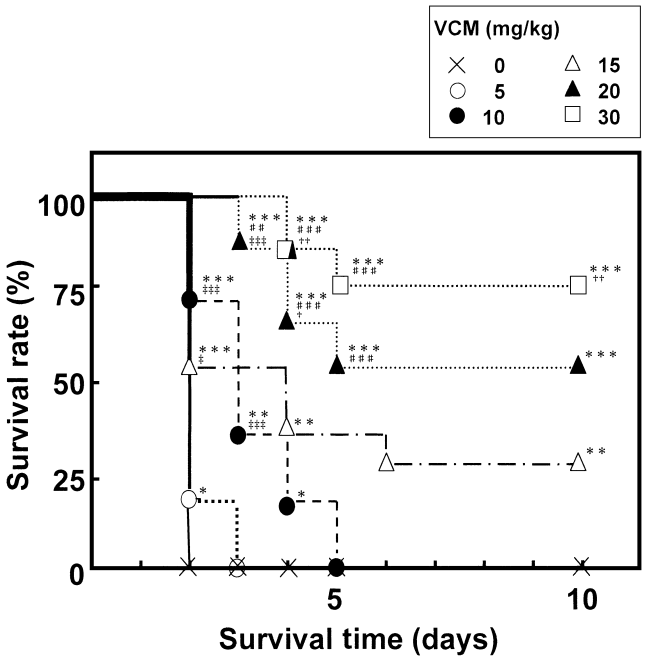

Fig. 2. The survival rate of *L. monocytogenes*-infected mice under the treatment with VCM alone. The data of 20 mice were analyzed for significance by the x^2-test, compared with 0 mg/kg (**P*<0.05, ***P*<0.01, ****P*<0.001); compared with 5 mg/kg ($^{\ddagger}P$<0.05, $^{\ddagger\ddagger\ddagger}P$<0.001); compared with 10 mg/kg ($^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001); or compared with 15 mg/kg ($^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01).

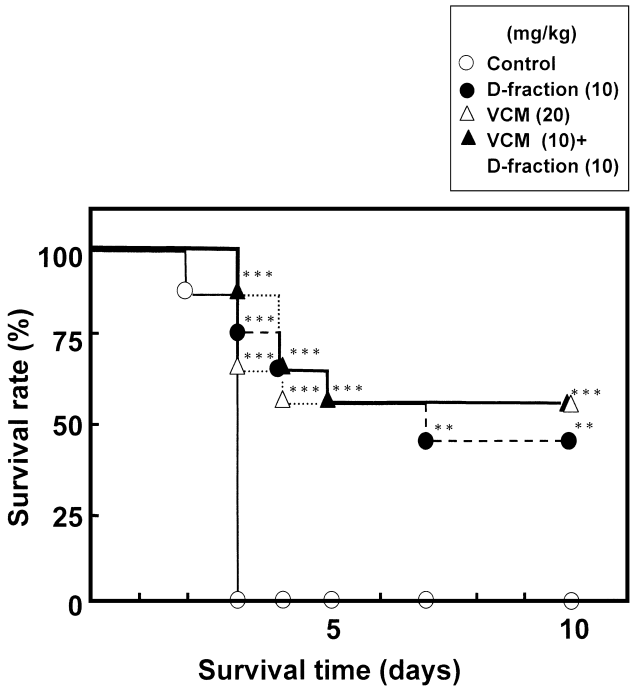

Fig. 3. The survival rate of *L. monocytogenes*-infected mice under the combined treatment with D-fraction and VCM. The data of 20 mice were analyzed for significance by x^2-test, compared with control (***P*<0.01, ****P*<0.001).

results demonstrate that VCM is effective on *Listeria*-infected mice at the range of 15 – 30 mg/kg per day. Therefore, we decided to use 20 mg/kg per day of VCM for the rest of the experiments.

Survival rate of L. monocytogenes-infected mice under the combined treatment with VCM and D-fraction

Figure 3 shows that all of non-treated control mice died by the 3rd day. In contrast, in the D-treated group (10 mg/kg per day) only 5 mice died by the 3rd day, and 8 still survived on the 10th day. In the VCM-treated group (20 mg/kg per day), 6 mice died by the 3rd day, and 12 survived on the 10th day. In the VCM+D-treated group (D-fraction: 10 mg/kg per day, VCM: 10 mg/kg per day), 2 mice died by the 3rd day, but 12 mice survived on the 10th day at the survival rate of 60%. Survival rate of the VCM+D-treated group is almost the same as that of the VCM-treated group. Furthermore, although all the mice, treated with 10 mg/kg per day of VCM alone, died by the 5th day (Fig. 2), administration of the D-Fraction improves the survival rate to 60%, which was sustained by the 10th day.

These results suggest the possibility that the D-fraction can reduce the effective VCM dosage against bacterial infection.

Living L. monocytogenes in the abdominal cavity after the treatments

Table 1 shows the survival rates of *L. monocytogenes* in the PBS washes from the mouse abdominal cavities, as described in Materials and Methods. In D-treated, VCM-treated and VCM+D-treated mice, the survival rates of these bacteria were 67%, 37% and 6% of non-treated control mice, respectively. These results indicate that bactericidal activities of the host were enhanced by pre-administration of the D-fraction.

Table 1. The survival of *L. monocytogenes* in peritoneal cavity

Treated groups	Numbers of colony
	Abdominal cavity ($\times 10^3$ CFU/ml) (ratio)
Control	4.03 ± 0.78 (1.00)
D-fraction	2.69 ± 0.95 (0.67)
VCM 20 mg/kg per day	1.50 ± 0.35 (0.37)
D-fraction + VCM 10 mg/kg per day	0.25 ± 0.55 (0.06)

D-fraction was administered at the concentration of 10 mg/kg per day. Each value represents the mean ± S.D. of 3 mice. Significant difference by Student's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001).

IL-1β production by peritoneal macrophages

To elucidate whether these effects of D-fraction were involved in macrophage-mediated reinforcement of the immune defense system, IL-1β production by peritoneal macrophages was also investigated. In the D-treated and VCM+D-treated group, IL-1β production was 2.6 – 2.7 times of that in the non-treated control group (Fig. 4), suggesting that the D-fraction activates the function of peritoneal macrophages. In addition, IL-1β production is also not influenced by VCM.

Bactericidal activities of splenic T cells against L. monocytogenes

To clarify whether T cell function is consequently enhanced by IL-1 production, bactericidal activities of splenic T cells were investigated (Table 2). When the CFU, obtained using splenic T cells of the control group mice, was set at 100%, the CFU in VCM-, D- and VCM+D-treated groups were reduced to 52%, 79% and 38% of that in the control group, respectively. These results revealed that bactericidal activity of splenic T cells was increased by D-fraction treatment.

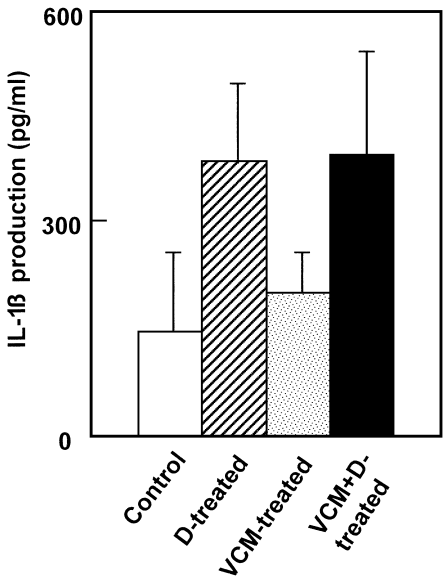

Fig. 4. Release of IL-1 under combined treatment with D-fraction and VCM. Each bar represents the mean ± S.D. of 3 mice.

Table 2. Bactericidal activitis of splenic T cells

Treated groups	Numbers of colony
	Splenic T cells ($\times 10^7$ CFU/ml) (ratio)
Control	3.29 ± 0.85 (1.00)
D-fraction	2.59 ± 0.92 (0.79) *
VCM 20 mg/kg per day	1.71 ± 0.87 (0.52)
D-fraction + VCM 10 mg/kg per day	1.25 ± 0.93 (0.38) **

D-fraction was administered at the concentration of 10 mg/kg per day. Each value represents the mean ± S.D. of 3 mice. Significant difference by Student's *t*-test (*P<0.05, **P<0.01).

DISCUSSION

The opportunistic bacterial infection is one of the major clinical problems in the treatment for patients with high risks. Especially, the establishment of effective treatment for MRSA infection is urgently required. Although VCM is well known to be exclusively effective against MRSA infection, a variety of serious side effects by VCM was pointed out. A new clinical strategy that can support the VCM treatment, not only against MRSA but also against secondary bacterial infections followed by MRSA, was necessary.

Our previous findings have revealed that *maitake* D-fraction effectively reinforces the action of anti-cancer drugs in both clinical and experimental studies (7). This polysaccharide-rich fraction is suggested to exert its effects via activating immune systems of hosts, for example, triggering the production of interlukins from macrophages (data unpublished). Based on these findings, we have assumed that immunotherapy using the D-fraction can also be effective and supportable to the treatment with antibiotics for bacterial infections.

In the present study, addition of D-fraction with VCM markedly improved the survival rate of *Listeira*-infected mice (Figs. 2 and 3). Moreover, the number of living *Listeria* in the abdominal cavity was significantly reduced by the combined treatment with D-fraction and VCM, compared with that seen in the group treated with VCM alone (Table 1). These results suggest that the D-fraction can reduce the effective dosage of VCM for bacterial infections, at least in a experimental study using mice.

To address the mechanism underlying these effects of the D-fraction, we examined the function of peritoneal macrophages by measuring the production of IL-1β. As shown in Fig. 4, IL-1β production was significantly increased by addition of D-fraction and it was not influenced by VCM, indicating that the function of macrophages was activated by pre-administration of D-fraction. Increased IL-1β may enhance the killing activity of macrophages against bacteria inoculated in the abdominal cavity.

It is known that produced IL-1β activates not only functions of macrophages, but also the cytotoxicity of splenic T cells (11). Therefore, we next examined the bactericidal activity of splenic T cells, which were prepared from mice treated with or without D-fraction. As shown in Table 2, the bactericidal activity of T cells treated with D-fraction was strengthened, although it is not clear why

single administration with VCM also enhanced the activity. Taken together, these results suggest the involvement of activated macrophages and T cells in the reinforcement of VCM therapy by D-fraction.

In conclusion, our present study demonstrates that the addition of D-fraction can effectively reduce the dosage of VCM in the treatment of *Listeria* infection. This raises the possibility that immunotherapy using the D-fraction has a clinical benefit for the treatment of opportunistic bacterial infection, although further studies are needed to clarify whether combined therapy using D-fraction and VCM was clinically effective for the treatment of MRSA infection itself.

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