Abstract. Background/Aim: Bacterial lipopolysaccharide (LPS) is involved in the activation of the innate immune responses on monocytes/macrophages in vitro, and by intravenous injection. Although small quantities of LPS are usually found in traditional Chinese medicines, vegetables and fruits, the mode of action of orally administered LPS is still unclear. Materials and Methods: LPS derived from Pantoea agglomerans (LPSp) was orally administered to C3H/HeN or C3H/HeJ mice ad libitum. Results: The LPSp treatment enhanced phagocytosis by resident peritoneal macrophages of C3H/HeN mice but not of C3H/HeJ mice. This activation can be defined as primed activation because no augmentation of inflammatory cytokines production was detected. LPSp in peritoneal fluid was detected and successfully quantified. Moreover, the LPSp reduced the expression of avian reticuloendotheliosis viral oncogene-related B (RelB) in the macrophages without degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (IkBa). Conclusion: Orally administered LPSp can reach the peritoneum, and enhance phagocytosis via Toll-like receptor 4 signaling pathway in resident peritoneal macrophages.

Food drives many tertiary functions that maintain health, including immune system activities responsible for disease prevention and resistance. It is expected that these tertiary functions can be harnessed to counteract lifestyle-related diseases, such as hyperlipidemia, diabetes, and cancer, and solve problems of the aging population. Therefore, studies of functional food are crucial for overall human health.

Bacterial lipopolysaccharide (LPS) is an amphipathic molecule comprising of lipid A, a core polysaccharide, and an O polysaccharide as it main components (1). LPS has been extensively studied as a Gram-negative bacterial toxin; it is responsible for the induction of septic shock. Major events in the pathogenesis of sepsis include neutrophil, monocyte, and macrophage inflammatory responses, intravascular coagulopathy resulting from activation of plasma complements and clotting cascades, endothelial cell damage, and hypotension. Thus, LPS exerts pleiotropic effects on many tissues and organs and can cause multiple organ damage, circulatory collapse, and death (2).

Improvements in hygiene have reduced exposure to LPS and has been associated with an increase in allergenic diseases (3). Lack of exposure to LPS may adversely affect the immune balance in the body. Hrmcr et al. demonstrated that an LPS-rich diet drives the development of regulatory T-cells and Th1-type immune response activation (4). In our previous studies, a significant amount of LPS was found in traditional Chinese medicines, vegetables, cereals and fruits (5, 6). Some of these plants contained enough LPS (1-100 μg/g dry weight) to activate macrophages in vitro and in vivo. Montenegro et al. reported the significant role of LPS as macrophage-activating substances in Jujen-taiho-to (a Chinese herbal medicine used for boosting human immune functions) (7). These facts prompted us to consider the macrophage-activating effect by oral administration of LPS.

The symptoms of dextran sodium sulfate (DSS)-induced colitis are more severe in mice with knock-out of Toll-like receptor (Tlr) 2, Tlr4, and myeloid differentiation primary response gene 88 (MyD88) than in wild-type mice; MyD88 is an adapter protein involved in signal transduction by TLRs (8). LPS stimulates α-defensin production in a TLR4-dependent manner in Paneth cells, which provides host defenses against microbes in the small intestine (9). Fukasaka et al. demonstrated that sublingual administration of LPS from Pantoea agglomerans with influenza vaccine promoted anti-influenza IgA production on various mucosa.
via the TLR4 pathway (10). These results suggest that LPS contributes to regulating homeostasis and activating macrophages through TLR4 signal cascade of the intestinal and oral mucosa. However, the mode of action in tissues other than the mucosa after oral administration of LPS is still unclear. We previously demonstrated that orally administered LPS of *Pantoea agglomerans* which was isolated from wheat flours (11) increased expression of tumor necrosis factor α (TNFα) in serum of mouse and teleost fish (12). The aim of this study was to clarify the effect of oral administration of a low dose of LPS on primed activation of resident peritoneal macrophages and phagocytosis.

**Materials and Methods**

*Animals and reagents.* Male C3H/HeN and C3H/HeJ mice were purchased from Japan SLC (Shizuoka, Japan). They were 6-8 weeks of age and weighed approximately 20 g at the start of the experiments. The animal experiments were approved by The Animal Care and Use Committee for Kagawa University (approval no. 139). The mice were given distilled drinking water containing LPS derived from a Gram-negative plant symbiotic bacteria, *Pantoea agglomerans* (LSPsp; Macrophi, Kagawa, Japan) (13), *ad libitum* for 7 days (n=5/group). The negative control mice received distilled water only (n=5/group). They were housed on a 12 h light-dark cycle and allowed unrestricted access to standard mouse chow. Mice were humanely euthanized on day 7.

*Phagocytosis assay.* A flow cytometric method was used for the phagocytosis assay. Peritoneal excluded cells (PECs) were harvested by flushing the peritoneal cavity of mice with 5 ml ice-cold phosphate-buffered saline (PBS). PECs were washed twice in PBS with 2% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). The cells were incubated with phagocytic particles in the form of fluorescent polystyrene latex beads (Polyscience, Warrington, PA, USA) or *Escherichia coli* (K-12 strain) BioParticles conjugated with Alexa Fluor 488 (Invitrogen) at 37°C, according to the manufacturer’s instructions. After incubation with the phagocytic particles, PECs were stained with Alexa Fluor 647- cluster of differentiation 11b (CD11b) antibody (M1/70, BioLegend, San Diego, CA, USA). Phagocytosis was evaluated by detecting Alexa Fluor 647 (CD11b) and phagocytic-particle double-positive cells using a Becton Dickinson FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Further phenotypic analysis of the CD11b-positive PECs were performed by additional staining with Alexa Fluor 488-B220 antibody (RA3-6B2; BioLegend) and phycoerythrin with cyanin-7 labeled F4/80 (PE-Cy7-F4/80) antibody (BM8; BioLegend). The populations of macrophages and B-1 cells were evaluated as CD11b+488/480+B220- cells and CD11b+F4/80+B220+ cells, respectively (14). As an isotype control, Alexa Fluor 488-IgG2b and PE-Cy7-IgG2a (BioLegend) were used. Data were analysed using FlowJo software (Ashland, OR, USA).

*Isolation of CD11b-positive peritoneal macrophages.* Resident peritoneal macrophages were isolated and purified from PECs by positive immunoselection using MACS® Anti-CD11b beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s instructions. The cell populations contained >80% CD11b+ cells by flow cytometric analysis.

**Immunoblotting and enzyme-linked immunosorbent assay (ELISA).** Resident peritoneal macrophages were isolated and purified as described above. The proteins were extracted by PRO-PREP (iNtRON Biotechnology, Sangdawon-Dong, Korea). Samples were electrophoresed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies after blocking and then were incubated with horsedarish peroxidase (HRP)-conjugated secondary IgG antibodies (Cell Signaling Technology, Tokyo, Japan) to nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (C-21; Santa Cruz Biotechnology, CA, USA), avian reticuloendotheliosis viral oncogene related B (RelB) (C-19; Santa Cruz Biotechnology), β-actin (Santa Cruz Biotechnology). Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (PerkinElmer, Kanagawa, Japan) on X-ray film. Levels of interleukin 6 (IL6), IL12p40 and tumor necrosis factor α (TNFα) in the culture supernatants of PECs before and after the phagocytosis test as described above were determined by an ELISA kit (BioLegend), according to the manufacturer’s instructions.

*Detection of orally administered LSPsp by specific ELISA.* The ELISA for quantifying LSPsp was based on a sandwich method. In brief, a plate coated with diluted capture antibody (34G2; Macroph) was incubated overnight at 4°C. The following day, the plate was washed with Tris-buffered saline containing Tween 20 (Wako, Osaka, Japan) and 3% bovine serum albumin (BSA; Invitrogen) in PBS added for 1 h to block non-specific binding. The plate was washed four times, and peritoneal fluids obtained from C3H/HeN mice were added and diluted standard LSPsp. After incubation for 1 h, the plate was washed four times and diluted detection antibody (4E11, Macroph) added. After incubation for 1 h, the plate was washed and biotin-conjugated anti-mouse IgG3 (BioLegend) was added. After incubation for 1 h, the plate was washed and streptavidin conjugated poly-HRP added (Thermo Fisher Scientific, Hvidovre, Denmark). After incubation for 1 h, the plate was washed and TMB substrate solution added (BioLegend). Finally, the absorbance at 450 nm was measured using a microplate reader (TEKAN, Kanagawa, Japan).

**Statistical analysis.** Statistical comparisons of the differences between treatments for other parameters were analysed by one-way or two-way ANOVA combined with the Tukey–Kramer post-hoc test. Statistical differences between two groups were analysed by unpaired Student’s t-test. A *p*-value <0.05 was considered statistically significant. All results are expressed as the mean±SEM. All analyses were performed with StatMate V (ATMS, Tokyo, Japan).

**Results**

*Orally administered LSPsp enhances phagocytosis by resident peritoneal macrophages.* Phagocytic cells in the CD11b-positive resident peritoneal phagocytes were analyzed by using flow cytometry. The detailed gating strategy is depicted in Figure 1A. After oral administration of LPS derived from *Pantoea agglomerans* (LSPsp) for 7 days to male C3H/HeN mice, the phagocytosis of CD11b-positive peritoneal phagocytes against *Escherichia coli* (*E. coli*) bioparticles was
enhanced in a dose-dependent manner (Figure 1B). To investigate whether the activation of phagocytosis is associated with LPSp absorption, LPSp-hyporesponsive C3H/HeJ mice and latex beads were used for phagocytosis assay. As shown in Figure 1C, orally administered LPSp (1 mg/kg/day for 7 days) significantly enhanced phagocytosis in cells from C3H/HeN (LPS-responsive) mice but not from C3H/HeJ mice. It is well known that CD11b-expressing phagocytic innate immune cells are not only macrophages but also other cell types such as B-1 cells in the peritoneal cavity (15). Further phenotypic analysis of the PECs obtained from C3H/HeN mice treated with water showed that the CD11b+ cells, defined as macrophages, were predominant (64.5±2.3% of CD11b+ cells); the proportion of CD11b+B220+ cells, defined as B-1 cells, was 13.7±1.4%, and that of CD11b+F4/80+B220+ cells, containing other cells such as granulocytes, was 16.0±0.3%. In addition, LPSp (1 mg/kg/day) treatment did not significantly affect the population distribution of peritoneal cells (CD11b+F4/80+ cells, 66.5±4.1%; CD11b+B220+ cells, 10.4±1.8%; CD11b+F4/80+B220- cells, 19.1±2.9% of CD11b+ cells) (Figure 2).

**Determination of LPSp in peritoneal fluid.** We next examined the amount of LPSp in the peritoneal fluid of mice after oral administration of LPSp. We previously developed monoclonal LPSp-specific antibodies (16). Figure 3A shows...
that our established ELISA method successfully detected as low as pico-gram amounts of LPSp. After oral administration to mice of LPSp at 1 mg/kg/day for 7 days, 8.5 pg/ml of LPSp was detected in the peritoneal fluid (Figure 3B).

Orally administered LPSp induces primed activation of resident peritoneal macrophages. We further examined whether oral administration of LPSp (1 mg/kg/day for 7 days to C3H/HeN mice) affected cytokine production in resident peritoneal macrophages. As shown in Figure 4A, the LPSp treatment did not increase the production of inflammatory cytokines IL6, TNFα and IL12 compared with the water treatment. Recently, Deng et al. reported that a low dose of LPS, as low as 5 pg/ml, primes the expression of pro-inflammatory mediators in macrophages by reducing RelB (a regulator of cytokine transcription), thus enabling a more robust expression of cytokines in macrophages challenged with a second stimulation such as high-dose LPS (100 ng/ml), although 5 pg/ml of LPS alone does not trigger noticeable cytokine production in vitro (17). Thus, we further examined whether LPSp administration affected TNFα production in resident peritoneal macrophages after stimulation with E. coli bioparticles. As shown in Figure 4B, the macrophages from the LPSp-treated mice significantly increased their TNFα secretion in the culture supernatant after E. coli stimulation, although there was no significant difference before
stimulation. Moreover, the expression of RelB was reduced in the macrophages by LPSp administration (Figure 4C). Maitra et al. demonstrated that a low dose of LPS does not induce activation of nuclear factor κB pathway to prime macrophages (18). Our data also demonstrate that LPSp failed to cause IκBα degradation in macrophages (Figure 4C).

Discussion

Macrophages play crucial roles as sentinels in the first-line response to pathogens and as mediators of host defense (19). They also assist in maintaining homeostasis through immune regulation, e.g. by eliminating foreign substances and unwanted apoptotic cells (20, 21). Recent studies have reported that the resistance to viruses and bacterial pathogens was enhanced by sublingual administration of LPS (10). However, the biological role and the mode of action after oral administration of LPS is still unknown. In the present study, we evaluated the effect of orally administered LPSp on the phagocytic activities in resident peritoneal macrophages.

Firstly, we demonstrated that phagocytic activity in peritoneal phagocytes of C3H/HeN mice was enhanced in a dose-dependent manner after oral administration of LPSp. Because TLR4 is well known as a major receptor for LPS, we further examined whether the enhancement of the phagocytosis by the LPSp administration was dependent on the TLR4 signaling pathway. LPS-hyporesponsive C3H/HeJ mice possess a point mutation of Tlr4 (22) and were also used here. As expected, phagocytosis by peritoneal phagocytes obtained from C3H/HeJ mice was not increased after LPSp
treatment. Considering that the predominant immune cells of peritoneal phagocytes obtained here were CD11b+F4/80+ macrophages, these results suggest that the activation of phagocytosis in resident peritoneal phagocytes (macrophages) via TLR4 pathway may be elicited by LPSp absorption.

We next examined the presence of LPSp in peritoneal fluids after its oral administration. The Limulus amebocyte lysate (LAL) test is a widely used in vitro assay for detection of LPS (23). However, it has been reported that intravital LAL inhibitors can exert an adverse effect on the sensitivity of the LAL assay (24). It is also difficult to distinguish between administered LPSp and commensal LPS. We developed monoclonal antibodies specifically binding to LPSp O-antigen polysaccharides, which have a different structure from other LPS species (16). By using the ELISA method we previously established LPSp was detected in the peritoneal fluid at 8.5 pg/ml after oral administration of LPSp (1 mg/kg/day for 7 days). In vivo studies for analyzing the distribution of LPS have mainly focused on the intraperitoneal (i.p.) (25) or intravenous (i.v.) (26) routes. In addition, these studies used radioisotopes or fluorophore-labelled LPS that require expensive reagents and special equipment for the experiments. Since our established method for quantifying LPSp with high sensitivity was based on a conventional ELISA, this LPSp-ELISA method is a convenient quantifying assay for following the fate of LPSp in tissues/blood.

The present study firstly demonstrated that orally administered LPSp reached the peritoneal fluid, suggesting that LPSp may be absorbed via the gastrointestinal tract and then in part circulate systemically. Oral administration of LPS would exert different biological effects when compared to intraperitoneal or intravenous routes. The i.p. or i.v. injection of LPS was widely used for preparation of experimental animal model of sepsis to induce sublethal endotoxic shock (27), whereas oral administration of LPSp did not have a toxic effect on experimental animals. We demonstrated that a high dose of oral administration of LPSp (300 mg/kg for 28 days) had no evidence of hepatotoxicity, nephrotoxicity, inflammation and weight decrease in rats (28). Once LPS enters the bloodstream, some LPS would be transported and accumulate in many tissues such as the liver, kidney and spleen (26). Thus, further study focused on the detailed distribution and pharmacokinetics of LPSp in body after its oral administration is needed.

LPS at several pico grams per milliliter was thought to be enough to induce the primed activation of macrophages in vitro, whereas a high dose (>ng/ml) of LPS was expected to induce tolerant state. In the primed state, when macrophages were challenged with a second stimulation, such as high-dose LPS, the secretion of inflammatory cytokines was potently increased. In contrast, in the tolerant state, the production of cytokines was suppressed upon secondary challenge (17). Therefore, we investigated whether the resident peritoneal macrophages were induced to be in a primed state after LPSp was orally administered. We confirmed that the secretion of TNFα was significantly enhanced by macrophages of LPSp-treated mice after the E. coli bioparticle addition. Before the stimulation, no augmentation of cytokine (IL6, TNFα and IL12) production was found. These data suggest that resident peritoneal macrophages may be induced to undergo primed activation by low-dose LPSp administration via the oral route.

Our immunoblotting analysis demonstrated that the LPSp treatment preferentially reduced the expression of RelB without IkBα degradation in resident peritoneal macrophages, consistent with recent reports using mouse bone marrow-derived macrophages with pretreatment using low-dose LPS (17, 18). RelB is involved in a negative feedback mechanism for suppressing inflammatory cytokine expression and the degradation of RelB is dependent on TLR4-mediated pathway (17). Islam et al. also demonstrated that alveolar macrophages primed by LPS pretreatment enhanced the phagocytosis of E. coli bioparticles (29), suggesting LPS priming may be a beneficial effect on the maintenance of proper homeostatic balance by facilitating clearance of foreign substances. Taken together, these data suggest that orally administered LPSp reaches the peritoneal fluid, and that LPSp induces priming of the resident peritoneal macrophages, leading to the enhancement of innate host defense systems, such as phagocytosis.

To the best of our knowledge, this is the first report on the mode of action of orally administered LPS. Our findings provide new insight into the physiological properties of LPS, which, for a long time, has only been considered an endotoxin.

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