Induction of Nitric Oxide Production in RAW264.7 Cells under Serum-free Conditions by *O*-antigen Polysaccharide of Lipopolysaccharide

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Abstract. *Background: Lipopolysaccharide derived from Pantoea agglomerans (LPSp) mainly consists of two aggregates, the high-molecular aggregate (HMM-LPSp) and the low-molecular aggregate (LMM-LPSp). The structural differences between HMM-LPSp and LMM-LPSp seem to depend on the length of the O-antigen polysaccharide because the lipid A regions of the two fractions are quite similar. In this study, we examined the biological activity of LPSp focusing on the O-antigen polysaccharide using HMM-LPSp and LMM-LPSp under serum-free conditions. Materials and Methods: The binding of LPSp to RAW264.7 cells under serum-free conditions was analyzed by flow cytometry using LPSp-conjugated fluorescein isothiocyanate (FITC-LPSp). The biological activities of HMM-LPSp and LMM-LPSp under serum-free conditions were evaluated by the nitric oxide production. Results: FITC-LPSp showed higher fluorescence intensity under serum-free than serum-containing conditions. HMM-LPSp induced higher nitric oxide production than LMM-LPSp under serum-free conditions. Conclusion: The present study indicates that the reactivity of LPSp is affected by the O-antigen polysaccharide under serum-free conditions.*

Recently, it was noted that food drives many tertiary functions that regulate health, including immune regulation, which may prevent certain diseases or maintain health. In developed countries, it is hoped that these tertiary functions can be harnessed to counteract lifestyle-related diseases, such as hyperlipidemia, diabetes and cancer, and problems related to an ageing population. Therefore, the study on functional foods is a crucial subject for human health and happiness. With this in mind, we focused on the function of lipopolysaccharide (LPS), which is usually contained in healthy food (1).

LPS is an amphipathic molecule, and mainly consists of lipid A, a core-oligosaccharide, and an *O*-antigen polysaccharide. Although the majority of LPS-related pro-inflammatory and toxic functions originate in the lipid A moiety (2), some of the biological activities can be modified by structural elements in the core oligosaccharide (3). In addition, the repeating oligosaccharide subunit composition of the LPS *O*-antigen polysaccharide and its exposed position on the bacterial cell surface also appear to contribute to its biological activities (4-12). Previous reports suggested that immune activations could be modified by the *O*-antigen polysaccharide in LPS, and this phenomenon appears to be due to the chain length of the *O*-antigen polysaccharide. However, a previous study stated that the *O*-antigen polysaccharide in LPS can elicit a signal network after the interactions between this polysaccharide and serum transporter proteins such as the LPS-binding protein (LBP) (13-15). Thus, possible direct interactions of the *O*-antigen polysaccharide in LPS with cells remain unclear.

Here, we investigated the direct actions of the *O*-antigen polysaccharide of LPS derived from *Pantoea agglomerans* (LPSp) using the high-molecular LPSp aggregate (HMM-LPSp, long sugar chain) and low-molecular LPSp aggregate (LMM-LPSp, short sugar chain) under serum-free conditions in the absence of transporter proteins.

Materials and Methods

Cell line and culture conditions. The mouse macrophage-like cell line RAW264.7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells were cultured in 25-cm^2 flasks (Corning, Tokyo, Japan) with Dulbecco's modified Eagle's medium (D-MEM; Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS;

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Key Words: Pantoea agglomerans, LPS, LPSp, *O*-antigen polysaccharide, serum-free, HMM, LMM.

Moregate Biotech, Tokyo, Japan), and penicillin/streptomycin sulfate (Sigma-Aldrich, Tokyo, Japan). The cells were maintained at 37˚C in a CO_2 incubator (Sanyo, Gunma, Japan) and passed to a new culture flask (1×10^5 cells/ml) every three to four days when cultures reached 1×10^6 cells/ml.

Preparation of high- and low-molecular mass LPS. LPSp (hot phenol-extracted, Macrophi, Kagawa, Japan) was solubilized by 3% sodium deoxycholate (Sigma-Aldrich), 0.2 M NaCl (Nakalai Tesque, Kyoto, Japan), 5 mM-2Na (Dojindo, Kumamoto, Japan), 20 mM Tris-HCl (Nakalai Tesque) adjusted to pH 8.3. High- and low-molecular mass LPSp fractions (HMM-LPSp and LMM-LPSp) were separated by gel filtration chromatography on a Sephacryl s-200HR (GE Healthcare, Tokyo, Japan) in 0.25% (wt/vol) sodium deoxycholate, 0.2 M-NaCl, 5 mM-EDTA, 10 mM-Tris HCl (pH 8.3). LPSp fractions were dialyzed against distilled water (Otsuka Pharm, Tokushima, Japan) for removing deoxycholate and further purified by ethanol precipitation. After ethanol precipitation, each pellet was lyophilized. The preparations were checked by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with silver staining using Silver stain II kit wako (Wako). The staining profile was previously reported (16, 17) and the average molecular mass of HMM-LPSp and LMM-LPSp was calculated as 5,00 and 52,500, respectively.

Flow cytometric analysis. Preparation of LPS derived from *Pantoea agglomerans* (LPSp) conjugated with fluorescein isothiocyanate (FITC-LPSp) was previous reported (18). RAW264.7 cells were incubated with FITC-LPSp (1, 10 μg/ml) in culture medium (serum-containing conditions) and FCS-free medium (serum-free conditions) for 30 min at 4˚C, respectively. After incubation, the cells were centrifuged (430 ×*g*, 10 min, 4˚C) and washed with ice-cold phosphate buffered saline (PBS; Sigma-Aldrich) twice. To analyze FITC-LPSp binding to subpopulations of RAW264.7 cells, the cell suspensions were analyzed by a flow cytometer (Cell Lab Quanta SC; Beckman Coulter, Tokyo, Japan) equipped with an argon-ion laser operating at 488 nm. Fluorescence intensity of each cell was recorded on a logarithmic scale and data were analyzed with Flowjo software (Tree Star, OR, USA). The results are expressed as the mean fluorescence of 5,000 cells.

Nitric oxide assay. RAW264.7 cells (2×10^5 cells/well) were cultured in 96 well plates (Beckton Dickinson, Tokyo, Japan) with 200 μl of culture medium and FCS-free media containing LPSp, HMM-LPSp and LMM-LPSp at 37˚C for 24 h in CO_2 incubator. After incubation, the plates were centrifuged (KUBOTA4000, Kubota, Tokyo, Japan) at 430 ×*g* for 10 min and the supernatant collected. The nitrite concentration in culture medium was measured as an indicator of nitric oxide (NO) production according to the Griess reaction using Griess reagent 0.1% *N*-(1-naphthyl) ethylenediamine (Sigma-Aldrich) in water and 1% sulfanilamide (Sigma-Aldrich) in 5% phosphoric acid (Nakalai Tesque), mixed 1:1. Concentrations of nitrate were estimated by absorbance reading at 550 nm (iMark microplate reader; BioRad, Tokyo, Japan) against standard solutions of sodium nitrite prepared in the same medium.

Statistical analysis. For statistical comparisons, two-way ANOVA with Bonferroni *post-hoc* tests were used. All statistical analyses were performed with Prism 5 software (Graphpad Software, San Diego, CA, USA). *p*-Values less than 0.05 were considered significant in this study.

Results

LPSp binds to RAW264.7 cells under serum-free conditions. To confirm that LPSp binds to macrophages under serum-free conditions, we performed a flow cytometric assay using RAW264.7 cells and LPSp conjugated to the FITC (FITC-LPSp), prepared as described previously (18). When RAW264.7 cells were incubated with FITC-LPSp in FCS-free medium, the frequency of fluorescent cells and fluorescence intensity in RAW264.7 cells was detected (Figure 1B and 1C). The frequency and fluorescence intensity was increased in a dose-dependent manner (Figure 1D and 1E). When the frequency of FITC-positive cells reached the maximum rate (100%) in both serum-free and serum-containing conditions, the fluorescence intensity of RAW264.7 cells incubated with FITC-LPSp under serum-free conditions was significantly higher than that under serum-containing conditions (Figure 1E).

These results indicate that LPSp can bind to RAW264.7 cells under serum-free conditions.

LPSp exhibits biological activity under serum-free conditions. Next, we investigated whether the binding of LPSp to RAW264.7 cells under serum-free conditions resulted in biological activity. NO production increased in a dose-dependent manner under serum-free conditions, but the induction of NO under serum-free conditions was lower than the one observed under serum-containing conditions (Figure 2). This result indicates that LPSp has a biological activity under serum-free conditions.

The O-antigen polysaccharide of LPSp exhibits biological activity under serum-free conditions. To verify the biological activity of the *O*-antigen polysaccharide of LPS under serum-free conditions, the NO assay was further performed using HMM-LPSp and LMM-LPSp. As mentioned earlier, LPS represents an aggregate of heterogeneous molecules, and the heterogeneity is influenced by structured differences in the *O*-antigen polysaccharide. As previously reported, aggregates of LPSp resolved by SDS-PAGE and silver staining mainly comprised of two aggregates: a high-molecular weight aggregate (molecular mass=32,500-72,500 Da, average 52,500 Da) and a low molecular weight aggregate (molecular mass=3,000-7,000 Da, average 5,000 Da) (14). Structural differences between HMM-LPSp and LMM-LPSp seem to depend on the length of the *O*-antigen polysaccharide because the lipid A regions of the two fractions are considerably similar. Thus, the involvement of the *O*-antigen polysaccharide of LPSp in mediating biological activities can be analyzed by comparing the activities of HMM-LPSp and LMM-LPSp. Importantly, as mentioned above, the molecular mass of HMM-LPSp and LMM-LPSp are undoubtedly different. The result was calculated by mole-based reduction. Considering molarity, HMM-LPSp induced greater NO production than LMM-LPSp (Figure 3).

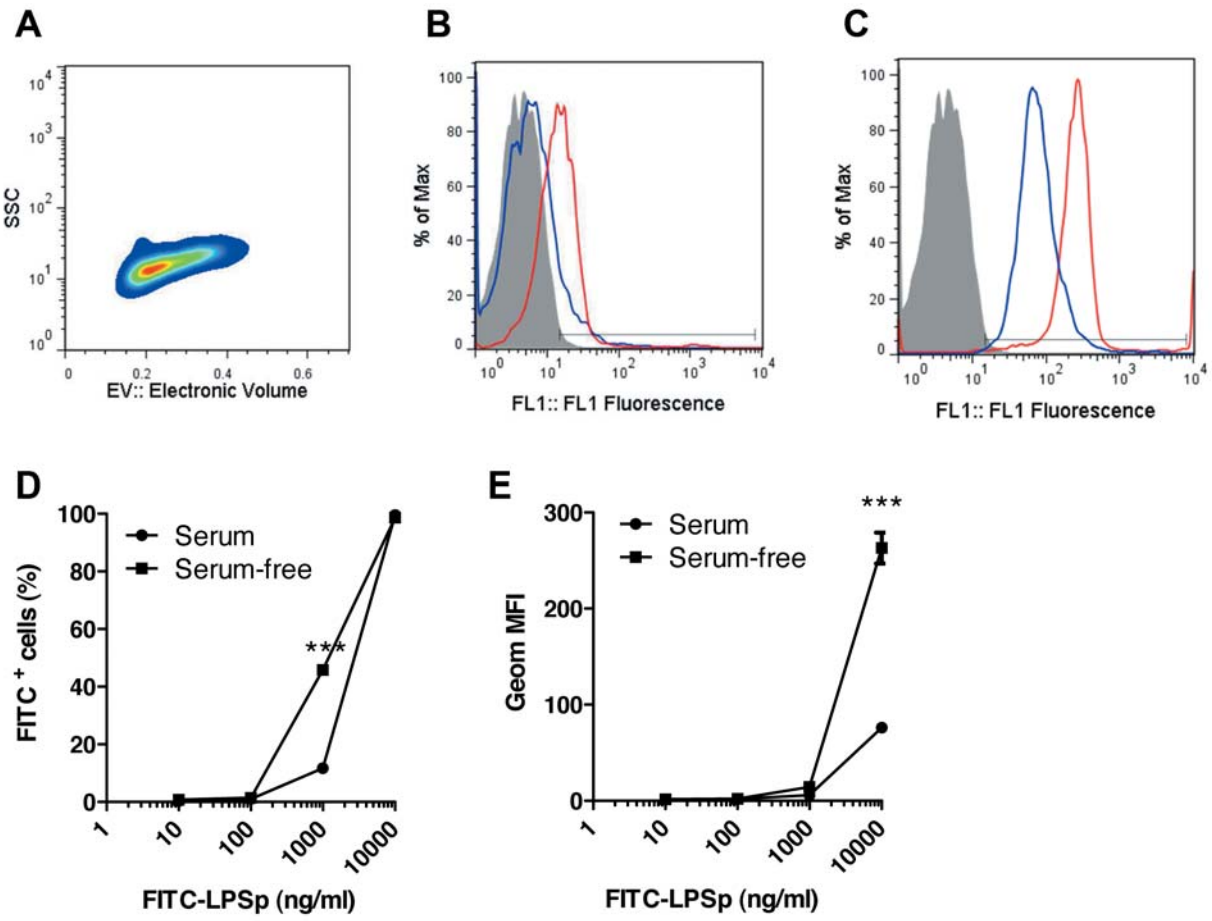

Figure 1. *Flow cytometric analysis of the binding of LPS derived from Pantoea agglomerans (LPSp) conjugated with fluorescein isothiocyanate (FITC-LPSp) to RAW264.7 cells under serum-free conditions. RAW264.7 cells were treated with LPSp or FITC-LPSp for 30 min at 4˚C. The cells were centrifuged and washed. The cells suspensions were analyzed by flow cytometry. A: Electric volume (EV)/side scatter (SSC) plots on RAW264.7 cells used in this study. B: Fluorescence histograms of RAW264.7 cells after treatment with LPSp 1 μg/ml (gray-filled, negative control), FITC-LPSp 1 μg/ml under serum-containing conditions (blue line) and FITC-LPSp 1 μg/ml under serum-free condition (red line). C: Fluorescence histograms of RAW264.7 cells after treatment with LPSp 10 μg/ml (gray-filled, negative control), FITC-LPSp 10 μg/ml under serum-containing condition (blue line) and FITC-LPSp 10 μg/ml under serum-free condition (red line). D: The frequency of FITC-positive cells in RAW264.7 cells after treatment. E: Geometric mean fluorescence intensity (Geom MFI) in RAW264.7 cells after treatment. Data are presented as the mean±SEM from quadruplicates, from at least three independent experiments (two-way ANOVA with Bonferroni post-hoc test; ***p<0.001).*

These results indicate that the *O*-antigen polysaccharide on LPSp is involved in the biological activity under serum-free conditions.

Discussion

In this study, we showed the biological activity of the *O*-antigen polysaccharide in LPS, using two aggregates HMM-LPSp and LMM-LPSp. LPS is well-known as the prototypical major pathogen-associated molecular pattern that is recognized by Toll-like receptor-4 (TLR4), in conjunction with the myeloid differentiation factor-2 (MD2), CD14, and serum proteins such as LBP. Importantly, serum LBP functions as a transporter of LPS to CD14 and to the TLR4 complex (19, 20). Thus, serum contains important elements for transducing cellular signals in response to LPS.

By flow cytometric analyses, FITC-LPSp was detected under serum-free conditions on RAW264.7 cells, suggesting that LPSp could bind to cells without the need for LBP. When the binding frequency reached 100%, fluorescence intensity of RAW264.7 cells incubated with FITC-LPSp was significantly higher under serum-free than serum-containing conditions. Therefore, we speculated that the binding and/or trafficking of LPSp under serum-containing and serum-free conditions were independently regulated. Phagocytic receptors [scavenger receptors, and complement receptors-

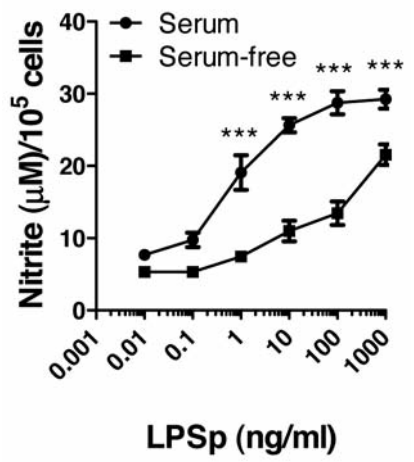

Figure 2. *Nitric oxide production in RAW264.7 cells incubated with LPS derived from Pantoea agglomerans (LPSp). RAW264.7 cells (2×10^5 cells/well) were incubated with LPSp under serum-free and serum-containing conditions for 24 h at 37˚C. The concentrations of released nitric oxide in the supernatant under each condition were measured using the Griess reagent. Data are presented as the mean±SEM from quadruplicates and are from at least three independent experiments (two-way ANOVA with Bonferroni post hoc test; ***p<0.001).*

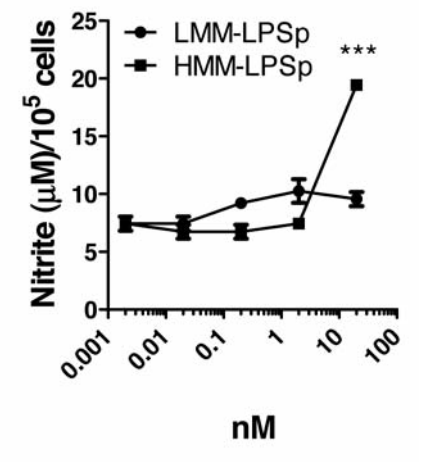

Figure 3. *Nitric oxide production in RAW264.7 cells incubated with high-molecular mass LPS derived from Pantoea agglomerans (HMM-LPSp) and low-molecular mass LPS derived from Pantoea agglomerans (LMM-LPSp). RAW264.7 cells (2×10^5 cells/well) were incubated with HMM-LPSp and LMM-LPSp under serum-free conditions for 24 h at 37˚C. The concentrations of released nitric oxide (as nitrite) in the supernatant under each condition were measured using the Griess reagent. Data are presented as the mean±SEM from quadruplicates, from at least three independent experiments (two-way ANOVA with Bonferroni post hoc test; ***p<0.001).*

3 (CR3) and CD11b/CD18] bind LPS (21-23). Thus, there may be at least two distinct mechanisms for LPSp binding and/or trafficking. These findings raise the question of whether binding and/or trafficking of LPSp under serum-free conditions results in biological activity. As shown in Figure 2, because LPSp induced NO production in RAW264.7 cells under serum-free conditions, we suggest that the binding and/or trafficking of LPS to the cells under serum-free condition did result in biological effects. Although the details of signal transduction will require further examination in future studies, here we assessed the involvement of the *O*-antigen polysaccharide in this biological activity. HMM-LPSp induced more NO production than LMM-LPSp on a *per mole* basis, suggesting that the *O*-antigen polysaccharide in LPSp imparts biological activity not only under serum-containing conditions, but also under serum-free conditions. These data undoubtedly show that the activities of LPS are not elicited by one-to-one molecular correspondence, but by depending on the form of the *O*-antigen polysaccharide and the molarity of LPS in solution.

We have previously reported that orally administered LPSp had positive effects such as phylactic, anti-allergic and antitumor effects (24). However, LPS has rarely been used clinically because intravenously administered LPSp behaves as a Gram-negative bacterial toxin that induces a variety of pro-inflammatory responses, leading to systemic inflammation, sepsis-related toxicity and septic shock. The effects of orally- and intravenously-delivered LPS are markedly different. The mechanism of action of orally administered LPS remains enigmatic, but the effects appear to be elucited by a mechanism different from the intravenous route, because LPS cannot be detected in the serum and does not induce cytokine production after oral administration of LPSp in mice (data not shown). The intravenous and oral routes of administration are analogous to serum-containing and serum-free conditions, respectively, suggesting that our study can be used as a starting point for analyzing the mechanisms of action of orally administered LPS. In addition, glycans are abundant and structurally diverse in the environment. Although long-studied, glycans are at the frontier of biological research.

In summary, our findings have shown that LPSp can bind to cells under serum-free conditions and that *O*-antigen polysaccharide in LPSp has certain biological activity under serum-free conditions. These findings may help improve our understanding over the mechanism of action of orally administered LPSp and other glycans.

Disclosures

The Authors have no financial conflicts of interest.

Acknowledgements

We thank the laboratory members in the Department of Immunology/Immunopathology, Faculty of Medicine, Kagawa University for helpful comments and constructive criticism in the experimental setup.

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Received April 4, 2013
Revised June 6, 2013
Accepted June 7, 2013