Functional Characterization of Lipopolysaccharide derived from Symbiotic Bacteria in Rice as a Macrophage-activating Substance

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Abstract. *Background and Aim: Lipopolysaccharide derived from a symbiotic bacterium in wheat (Pantoea agglomerans, LPSp) has shown multiple positive effects, such as prophylactic, antiallergic and antitumour effects, without serious side-effects. LPSp has differential biological activities in comparison to other LPS, such as those from Escherichia coli (LPSe). The only difference between LPSp and LPSe is in the O-antigen polysaccharide structure (O-PS). This led us to the hypothesis that the O-PS structure would seem to participate in biological activities. Thus, the characterization of properties of O-PS in LPS is of the utmost importance for understanding cell activation in the maintenance of homeostasis. However, little is known about the correlation between the O-PS structure of LPS and its biological activities. In this study, we extracted LPS derived from a symbiotic bacterium in rice (strain A46, related species of Pantoea), which has a long history of use in foods, and investigated its putative structures and functions. Materials and Methods: LPS derived from strain A46 was prepared using a hot phenol extraction method. The properties of LPS-A46 were analysed by thin-layer chromatography, Tricine SDS-PAGE and Western blotting. The function of LPS-A46 was analyzed by quantative real-time PCR and flow cytometry using THP-1 cells and Peripheral blood mononuclear cell (PBMC) derived macrophages. Results: In Tricine SDS-PAGE, high molecular mass LPS-A46 had a molecular mass lower than that of LPSp. In Western blotting, LPS-A46 reacted with lipid A antibody but did not react with an O-PS antibody of LPSp. In comparison to other LPS, LPS-A46 induced a differential cytokine gene expression profile in THP-1 cells and PBMC-derived macrophages. Conclusion: The present study suggests that LPS derived from symbiotic bacterium in rice is a bioactive functional LPS which may have different functional activities compared to other types of LPS.*

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Key Words: Pantoea agglomerans, A46, LPS, LPSp, LPSa46, macrophage, M-CSF, GM-CSF.

Antigen-presenting cells, such as monocytes, macrophage and dendritic cells, play a pivotal role as sentinels for first-line alerts and as mediators in host defense. These cells are dynamic and heterogenous due to different mechanisms, such as cytokines and microbial components, which profoundly affect the function of mononuclear phagocytes (1). Therefore, although macrophages are present in virtually all tissues, each macrophage type displays remarkable plasticity that allows it to efficiently respond to environmental signals and change its phenotype. Macrophages are also well known as the major cells of the leukocytic infiltrate of tumors. Tumor-associated macrophages (TAMs) originate as blood monocytes recruited from the tumor vasculature by tumor-derived signals (2-6). Compelling evidence has now suggested that the regulating activation of TAMs function is one of the key steps in tumor development and/or regression (7-10).

Bacterial lipopolysaccharide (LPS), which is generally considered to be an endotoxin, is the major constituent of the outer surface of Gram-negative bacteria. At very low concentrations (100 pg/ml), LPS is involved in the activation of innate immunity through monocytes or macrophages (11), which is important in a variety of diseases. Recent investigations have demonstrated an important observation, namely that exposure to LPS or bacterial infection in early life alters adult innate immune responses (12-17). The major findings from recent studies appear to show that activation of innate immunity not only provides host defence but is also important for maintaining homeostasis. However, clinical use of LPS has been limited because at high concentrations it acts as a Gram-negative bacterial toxin that can induce a variety of proinflammatory responses and thus lead to systemic inflammation and/or toxic functions that are usually related to sepsis and septic shock.

LPS is an amphipathic molecule that is composed of three parts: lipid A, core oligosaccharide and O-antigen polysaccharide (O-PS). It is well known that the majority of LPS-related proinflammatory responses and toxic functions are due to the lipid A moiety. However, some of these biological activities may be modified by structural elements of the contiguous core oligosaccharide (18). In addition, the repeating oligosaccharide subunit composition of the LPS O-side chain and its exposed position on the bacterial cell surface also appear to contribute to the virulence of bacterial LPS, as well as its immunogenicity. Much of the early interest in the chemistry, biosynthesis and genetics of O-PS originated from its roles as essential virulence determinant and its potential applications in the development of vaccines. These studies strongly suggested that O-PS in LPS was involved in host biological activity. Thus, changing the toxicity and/or biological activity of LPS by modifying its O-PS structure could result in a potentially safe molecule that is capable of activating innate immunity for preventing various diseases.

Food provides both nutrients and many tertiary functions for regulating health. Immune regulation by these tertiary functions may prevent certain diseases or maintain and augment health. In advanced countries, it is hoped that these tertiary functions can be developed to counteract the problems associated with lifestyle-related diseases such as hyperlipidaemia, diabetes and cancer, and problems related to the transition to an ageing society. This is based on the presence of LPS in some foods and its role in regulating immunity (19). In fact, we have previously reported that orally and/or intradermally administered LPS derived from *Pantoea agglomerans* (LPSp), which is a symbiotic bacterium in wheat, had positive effects, such as prophylactic, antiallergic and antitumour effects, without having serious side-effects (20). Thus, orally and/or intradermally administered LPS in food could induce activation of innate immunity. In addition, the only difference between LPSp and LPS derived from *Escherichia coli* (LPSe) is in the O-PS structure, which led us to hypothesize that O-PS structure would appear to participate in biological activities. Hence, a categorization of correlation between O-PS structure and its biological activities is required in order to understand the mechanisms of host-LPS interactions that are involved in maintaining homeostasis. However, little is known about the correlation between LPS derived from food and host biological activities *in vitro* and *in vivo*.

In this study, we extracted LPS derived from a symbiotic bacterium in rice (LPS-A46), which has a long history of use in foods, and investigated its putative structures and functions against human macrophages derived from THP-1 and human peripheral blood monocytes (PBMCs).

Materials and Methods

Bacteria strain. Symbiotic bacteria in rice (A46 strain), which is a species related to *Pantoea* was kindly given by Dr. Atsuko Ueki (Department of Bioprocess Engineering, Faculty of Agriculture, Yamagata University).

Reagents. Reagents were as follows: phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Tokyo, Japan), recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), human macrophage colony-stimulating factor (M-CSF) (PeproTech, NewJersey, USA), LPS derived from *Salmonella minnesota* R595 (List Biological Laboratories, CA), *Pantoea agglomerans* (LPSp; Macrophi, Takamatsu, Japan), *E. coli* (LPSe) and *Porphyromonas gingivalis* (Invivogen, Kyoto, Japan).

Preparation and purification of LPS derived from A46 strain. Crude extract of A46 strain was prepared by using a hot phenol extraction procedure, and then the crude extract was purified as described previously (21). All LPS-A46 preparations used in this report were >97% purified. The LPS content was calculated from dry weight, considering the nucleic acid and proteins estimated by absorbance and BCA protein assay kit (Pierce, Rockford, IL, USA) in the solution before drying. LPS-A46 preparation was tested with a LAL assay (Endospecy; Seikagaku Co, Tokyo, Japan) according to the manufacturer's instructions and calibrated with LPSe as standard.

Gel filtration chromatography. LPS-A46 was injected onto a TSK-gel G3000SW column (TOSOH, Tokyo, Japan). The column was pre-equilibrated with 100 mM ammonium acetate buffer (pH 5.0) at a flow rate of 1 ml/min. The fractions eluting from the column were monitored at 210 nm.

Tricine SDS-PAGE and Western blot analysis of LPS. LPS samples were separated by *N*-[2-hydroxy-1,1-*bis*(hydroxymethyl) ethyl glycine (Tricine) SDS-PAGE as previously described (22). Electrophoresis was performed at a constant 25 mA for approximately 1.5 h and visualized by silver staining II kit (Wako, Osaka, Japan). After electrophoresis, For immunostaining, LPS samples were transferred to PVDF filter (Biorad, Tokyo, Japan) and incubated overnight at 4˚C in the ECL blocking agent (GE Healthcare, UK, Ltd). The filter was probed with a monoclonal antibody against synthetic lipid A in *E. coli* (Clone 26-5; Abcam,

Cambridge, UK) and O-PS monoclonal antibody against *P. agglomerans* (Clone 4E11; Macrophi, Takamatsu, Japan) as primary antibody, respectively. And then, membrane was subsequently washed, incubated with an alkaline phosphatase-conjugated antibody, and developed using 5-bromo-4-chroro-3-indolyl phosphate (BCIP; Wako, Osaka, Japan) and nitro-blue tetrazolium (NBT, Wako).

Preparation of macrophages. THP-1 cell line (human acute monocytic leukemia cell line) was obtained from the American Type Culture Collection (ATCC, MD, USA). Cells were cultured at 0.5-5×10^5 cells/ml in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS; Moregate Biotech, Tokyo, Japan), penicillin and streptomycin sulfate (Invitrogen, Tokyo, Japan) and 2-mercaptoethanol (final conc. 50 nM). THP-1 cells were differentiated into different cell types according to the method described by Park *et al*. (23). Briefly, THP-1 cells were differentiated into different cell types in 60 mm dishes with 5 ml of the RPMI-1640 medium containing 5 ng/ml PMA over 48 h (because it is reported that this dose does not affect the cytokine gene expression). The dishes were washed three times with 5 ml PBS and the unattached cells were centrifuged, resuspended in medium, and counted in order to determine the adherence. Adherent cells were used for the subsequent experiments (as PMA-THP-1 cells).

PBMC-derived macrophages were obtained according to the methods described previously, with slight modification (24). Briefly, Ficoll-Hypaque-separated PBMCs were seeded at 10^6 cells/400 μl into 24-well plates in RPMI-1640 containing 10% FCS with recombinant human GM-CSF (final conc. 10 ng/ml) or M-CSF (10 ng/ml). On day 3, the medium was change and non-adherent cells were removed. The resultant cell layer was uniformly adherent with macrophage morphology.

Flow cytometric analysis. Adherent cells were washed with PBS and detached from the plastic by thorough scraping with a rubber policeman. PMA-THP-1 cells were washed in PBS with 0.5% FCS, incubated with appropriate antibodies, and then analyzed by flow cytometry using a Becton Dickinson FACS Calibur (Becton Dickinson, CA, USA). The following antibodies were used: anti-CD14 conjugated with FITC (clone MY4; Beckman Coluter, Tokyo, Japan), anti-CD11c conjugated with allophycocyanin (APC) (clone 3.9; eBioscience, San Diego, CA, USA), anti-CD209 (DC-SIGN) conjugated with APC (clone DCN46; BD Pharmingen), anti-CD86 conjugated with PE (clone IT2.2; BD Pharmingen), anti-HLA-DR conjugated with FITC (clone Immu-357; Beckman Coulter), anti-CD11b conjugated with FITC (clone IM0530; Beckman Coulter) and anti-TLR4/MD2 conjugated with PE (Santa Cruz, CA, USA). Data were analyzed using FlowJo software (Tree Star, OR, USA).

Quantitative real-time reverse transcription (RT) PCR analysis. Total cellular RNA of differentiated macrophages was extracted using RNeasy mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. RNA samples were reverse-transcribed using ReverTra Ace-α (Toyobo, Osaka, Japan). Real-time quantitative PCR with primer combinations (25, 26) was performed using iQ SYBR Green Supermix (Bio-Rad, Tokyo, Japan) according to the manufacturer's instructions. Primers for cyclophilin in humans were used as an internal control (27). All qRT-PCR reactions were run in duplicate, and the average Ct value was calculated. Amplification and detection were performed in an Opticon 3 monitor (Bio-Rad). PCR conditions were: 1 cycle of 95˚C for 10 min, 40 cycles of 95˚C for 10 s, 60˚C for 20 s and 72˚C for 20 s.

Table I. *The composition of the purified extracts of A46 strain*.

	Content
DNA	<2%
Protein	<1%
β-Glucan	<10^{-5}%
Limulus activity	150%

The composition of purified extracts were estimated by absorbance, bicinchoninic acid methods, Fungitec G test and Limulus test for nucleic acid, protein, β-glucan and LPS, respectively. The tests were performed essentially as recommended by the manufacturer's instructions as described in the Materials and Methods.

Lymphocyte activation assay. PBMCs were incubated with LPSp, LPSe and LPS-A46 for 6 h. After incubation, the cells were stained with anti-CD69 conjugated with FITC (clone FN50; BD Pharmingen) and anti-CD3 conjugated with PE-Cy5 (clone UCHT1; Beckman Coulter), and analyzed by flow cytometry as described above.

Statistical analysis. For statistical comparisons, data were analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison *post hoc* test. A p-value of <0.05 was considered significant.

Results

Lipopolysaccharide derived from strain A46 shows limulus activity and has a micelle structure. As shown in Table I, the extracts of strain A46 were >97% pure as calculated from the dry weight considering the nucleic acid, protein and β-glucan contents. LPS-A46 showed *limulus* activity (150%) when calibrated with LPS derived from *E. coli* (LPSe) as a standard using the Endospecy kit. The peaks detected by HPLC gel-filtration analysis at UV 210 nm for extracts of strain A46 and LPS derived from *Pantoea agglomerans* (LPSp) showed the same retention times (Figure 1A).

High molecular mass LPS-A46 showed a molecular mass lower than that of LPSp. Alterations or heterogeneity of LPS structure is most often assessed by alterations in electrophoretic band profiles using SDS-PAGE. To obtain a better resolution of LPS, tricine was used in the electrophoresis buffer instead of glycine. We investigated the molecular mass profile of LPS-A46 using Tricine SDS-PAGE and compared this with that of other LPS. LPS-A46 mainly showed two bands after visualization with silver staining (Figure 1B). High molecular mass (HMM, over the 30kDa molecular weight) LPS-A46 had a molecular mass lower than that of LPSp, whereas low molecular mass (LMM, 7kDa molecular weight or less) LPS-A46 had a molecular mass similar to that of LPSp.

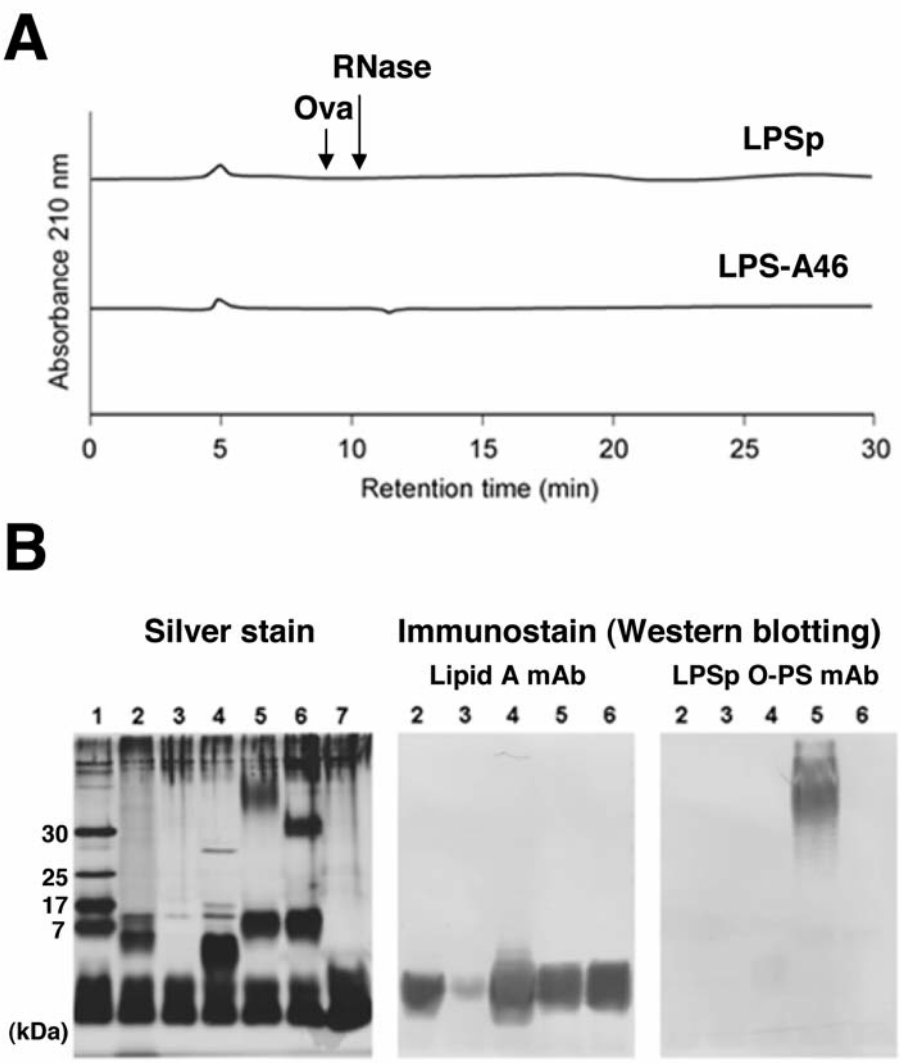
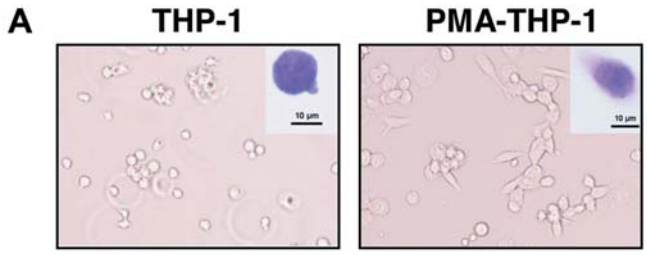
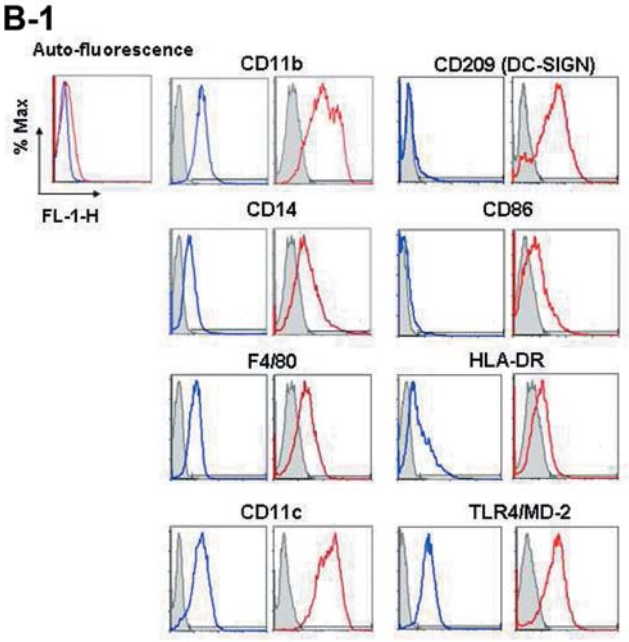


Figure 1. *A: Elution profile of LPSp and LPS-A46 gel filtration chromatography using HPLC. Each LPS (20 μg) was injected onto a column. The molecular weight of ovalbumin (OVA) and ribonuclease (RNase) are indicated by arrows. B: Silver and immunostained Tricine-SDS-PAGE profiles of LPS. Lane 1: Molecular weight marker, lane 2: Escherichia coli, lane 3: Porphyromonas gingivalis, lane 4: Salmonella minnesota R595, lane 5: Pantoea agglomerans. lane 6: A46 strain 7: sample buffer. LPS sample (1 μg) was separated by Tricine SDS-PAGE and stained with silver staining commercial kit. In the case of immunostaining, the gels were transferred to PVDF filter, and incubated overnight at 4˚C in the blocking agent. The membrane was subsequently washed, incubated with an anti-lipid A monoclonal antibody or anti-LPSp O-PS-specific antibody as primary antibody. The bands were developed by NBT/BCIP.*

The O-PS structure in LPS-A46 was different from that in LPSp. On Western blotting, all LPSs showed bands when reacted with an anti-lipid A monoclonal antibody at locations of LMM because this binds to synthetic and/or free lipid A molecules (Figure 1B). This indicated that the structure of lipid A is highly conserved among bacterial species. Previously, we prepared a specific monoclonal antibody against O-PS [clone 4E11, (18)] and used this to assess cross-reactivity. None of the LPS used in this study showed the band except for LPSp (Figure 1B).

LPS-A46 augments cytokine gene expression and induces different expression profiles for different cell types compared to other LPSs. Macrophages display remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype. Thus, it is possible that the response to LPS is different in each macrophage. In a pilot study, we analyzed the cytokine mRNA expressions in THP-1 and PMA-THP-1 cells following LPS challenge. As shown in Figure 2A and B (B-l, B-2), the THP-1 cells treated with PMA became adherent, showed spreading, and had high phagocytic





B-2

Difference between THP-1 and PMA-THP-1

	THP-1	PMA-THP-1
Adherence	0%	91-100%
Phagocytosis (latex beads)	25-30%	90-95%
CD11b	92.3%	82.3%
CD11c	89.3%	98.8%
CD14	34.1%	41.2%
CD86	5.30%	21.2%
F4/80	71.7%	38.0%
DC-SIGN	9.67%	78.1%
HLA-DR	39.3%	11.0%
TLR4 / MD2	92.7%	80.3%

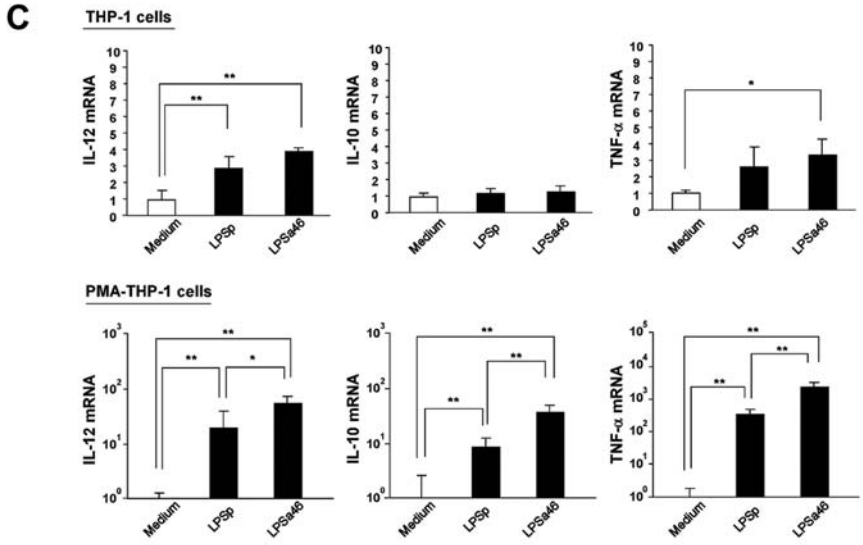


Figure 2. *A: Microscopy images of THP-1 and PMA-THP-1. Panels show the cells stained with Giemsa. B-1: THP-1 and PMA-THP-1 were stained with anti-CD11b, anti-CD14, anti-F4/80, anti-CD11c, anti-CD209, anti-CD86, anti-HLA-DR, and anti-TLR4/MD-2, and analyzed by flow cytometry (THP-1: blue line, PMA-THP-1: red line, isotype control: filled with gray). B-2: The data shows the percentage of positive cells, adherence and phagocytosis against latex beads in THP-1 and PMA-THP-1. C: Gene expression analysis of THP-1 and PMA-THP-1. THP-1 and PMA-THP-1 were incubated with LPSp (100 ng/ml) or LPS-A46 (100 ng/ml) for 4 h. The expression of IL-12, IL-10 and TNF-α was relative to that of the housekeeping gene (cyclophilin) by real-time PCR. Data represent the mean±SEM of quadruplicates. Asterisks indicate significant differences (*p<0.05, **p<0.01).*

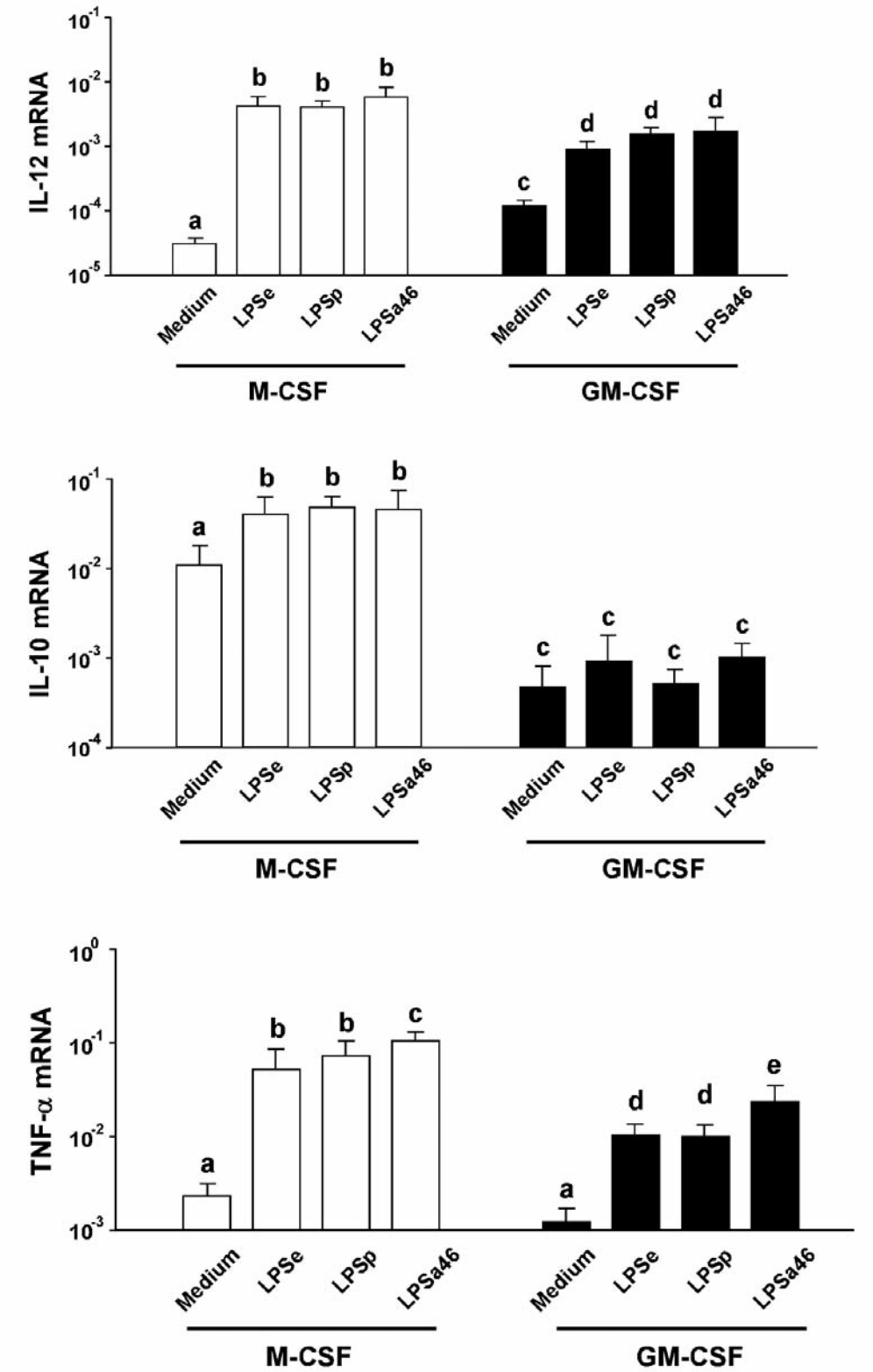


Figure 3. *Gene expression analysis of PBMC-derived macrophages using M-CSF and GM-CSF (M-PBDM, GM-PBDM). M-PBDM and GM-PBDM were incubated with LPSe, LPSp, and LPSa46 for 4 h (100 ng/ml). The expression of IL-12, IL-10 and TNF-α relative to that of the housekeeping gene (cyclophilin) by real-time PCR is shown. Data represent the mean±SEM of quadruplicates. Columns with different letters are significantly different ($p<0.05$).*

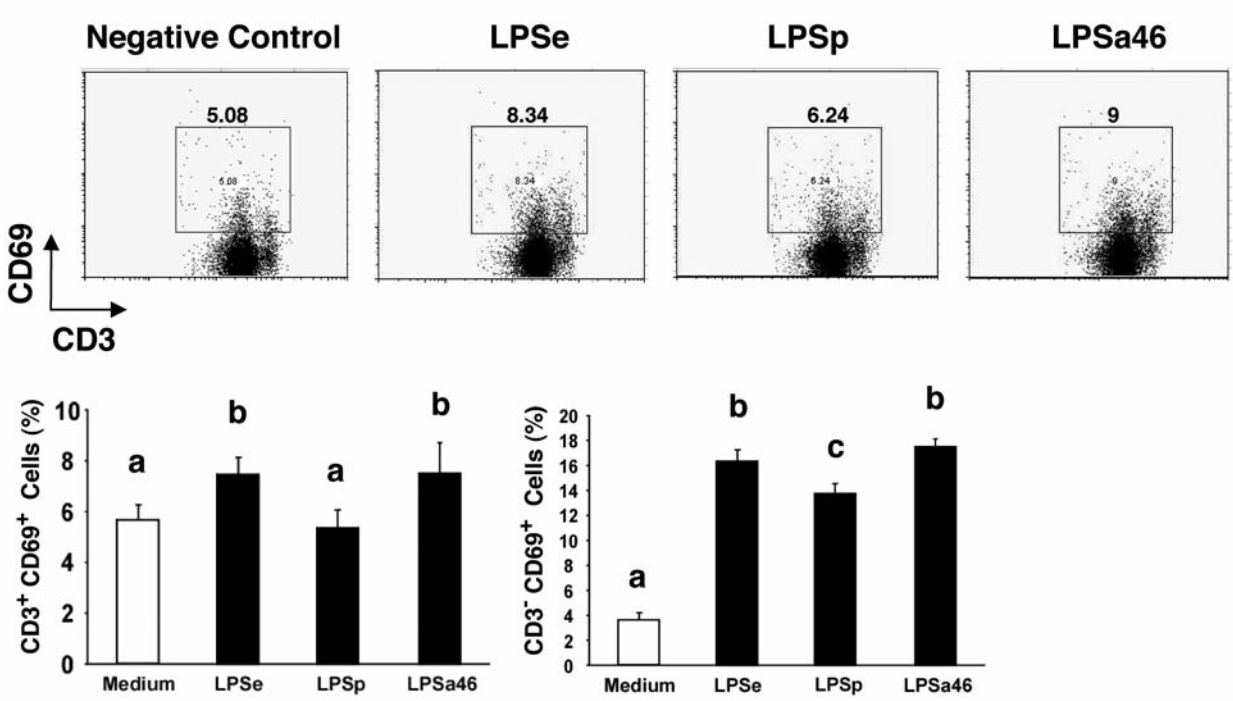


Figure 4. *Effect of LPS-A46 on lymphocyte activation. PBMCs were incubated with LPSe, LPSp, LPS-A46 for 6 h (1 μg/ml). After 6 h, the cells were stained with anti-CD3 and anti-CD69 monoclonal antibody and analyzed by flow cytometry. Data represent the mean±SEM of quadruplicates. Significant differences are indicated by different letters (p<0.05).*

activity. Comparing THP-1 and PMA-THP-1 cells, both expressed macrophage markers such as CD11b and F4/80 (this antibody is usually used against mouse, but clone BM8 has cross-reactivity against human monocytes/macrophages; Figure 2B). In addition, they also expressed CD14, TLR4/MD2, HLA-DR and CD11c. PMA-THP-1 cells had higher expression of the macrophage and DC markers such as CD209 (DC-SIGN) and CD86 (B7-2). These results indicate that THP-1 and PMA-THP-1 cells are different cell types (monocytes, macrophages or dendritic cells). For THP-1 cells, LPS-A46 significantly augmented *TNF-α* and *IL-12* mRNA expression but not that of *IL-10*. Furthermore, LPSp significantly augmented *IL-12* mRNA expression but not that of *TNF-α* and *IL-10*. However, the augmented expression was minimal. In PMA-THP-1 cells, both LPSp and LPS-A46 significantly augmented *TNF-α, IL-12* and *IL-10* mRNA expression. This augmentation was higher than that in THP-1 cells. Furthermore, LPS-A46 showed a higher induction of *TNF-α* and *IL-10* mRNA expression than that shown by LPSp.

Further analysis used PBMC-derived macrophages that were cultured *in vitro* for 7 days in the presence of GM-CSF or M-CSF. The adherent cells (GM-PBDM and M-PBDM cells, respectively) were collected and used for gene expression analysis and functional studies. Using real-time PCR analysis, the basal gene expressions of GM-PBDM and M-PBDM cells were different (Figure 3). M-PBDM cells had higher *IL-10* mRNA expression than GM-PBDM cells, whereas GM-PBDM cells had higher *IL-12* mRNA expression than M-PBDM cells. Thus, M-PBDM and GM-PBDM are possibly cells with different macrophage phenotypes.

LPS-A46 augmented *IL-12, IL-10* and *TNF-α* mRNA expression of M-PBDM cells. Although these augumentation were also observed using LPSp and LPSe, LPS-A46 augmented *TNF-α* mRNA expression in a manner that was significantly different from that of LPSp and LPSe. In GM-PBDM cells, all of LPSs used in this study augmented *IL-12* and *TNF-α* mRNA expression, whereas *IL-10* mRNA expression remained unchanged. Similarly to M-PBDM cells, LPS-A46 augmented *TNF-α* mRNA expression in a manner that was significantly different from that of LPSp and LPSe.

LPS-A46 induced CD69 expression. It is known that LPS is a potent mitogen for B-cells in mice and humans, and plays an important role in the generation of antigen-specific antibody responses. In addition, it is also known that CD69 is involved in the early events of T- and B-cell activation. Therefore, we investigated lymphocyte activation in the early and proliferation stages. As shown in Figure 4, LPS-A46 and LPSe significantly induced CD69 expression on $CD3^+$ cells, however LPSp did not induce CD69 expression on $CD3^+$ cells.

On the other hand, all LPS used in this study significantly induced CD69 expression on $CD3^-$ cells, but LPS-A46 and LPSe induced higher expression than that of LPSp.

Discussion

In the present study, we assessed the putative structures and functions of LPS derived from symbiotic bacteria in rice (strain A46). It is well known that LPS has a strong tendency to form micelles in solution due to its amphipathic nature, and smooth-type LPS is believed to self-assemble into micelle structures that have molecular masses of greater than 1 MDa (28, 29). This self-aggregation behaviour is derived from the characteristics of the lipid A component of the LPS molecule. The peaks for LPSp and extracts of strain A46 were detected at high molecular weight positions at the same retention times by HPLC gel-filtration analysis (Figure 1A). These results suggest that LPS-A46 has a lipid A function for forming micelle structures similar to that of LPSp and other LPS.

In Western blotting analysis, all LPS used in this study showed bands at locations of LMM because of the properties of the lipid A antibody used, which binds to synthetic and/or free lipid A molecule (Figure 1B). This result indicates that the structure of lipid A is highly conserved among bacterial species. However, the locations and staining concentrations of the bands appeared to be different, thus suggesting that each lipid A structure was slightly different. In particular, the band for LPS-A46 was more similar to that of LPSp than for any other LPS used in this study. This indicates that the lipid A structure of LPS-A46 is similar to that of LPSp. In addition, none of the LPS exhibited cross-reactivity, except for LPSp, when using an LPSp O-PS-specific antibody (Figure 1B), indicating that the O-PS structure of LPSp is different from that of any of the other types of LPS. Taken together, our results indicate that the lipid A of LPS-A46 is similar to that of LPSp but the O-PS structure is different. However, further detailed analysis with nuclear magnetic resonance and/or mass spectroscopy is needed.

The extract of strain A46 showed limulus activity (Table I). These results led us to assume that the extract of strain A46 was a bioactive functional LPS in host cells. To assess the functions of LPS-A46, we prepared several types of macrophages (THP-1, PMA-THP-1, M-PBDM and GM-PBDM cells). Macrophages display remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype. In a pilot study, we analysed the functions of LPS-A46 using THP-1 cultured cells and PMA-THP-1 cells.

Analysis by flow cytometry showed both THP-1 and PMA-THP-1 cells expressed CD11b, F4/80, CD11c, CD14, HLA-DR and TLR4/MD-2 markers on their surfaces. Most studies have used CD11c as a DC lineage marker, although there is no evidence for any direct function of CD11c (30). Furthermore, it has also been suggested that CD11c in humans is a marker for mononuclear phagocytes and an active complement receptor (CR4) that is induced during macrophage maturation (31, 32). Therefore, it appeared that recent studies did not support there being a distinction between macrophages and DCs.

PMA-THP-1 cells exhibited significantly higher phagocytosis and adherence than did THP-1 cells. In addition, PMA-THP-1 cells clearly expressed higher levels of CD86 and CD209 (DC-SIGN) on their surfaces. These results indicate that THP-1 and PMA-THP-1 are possibly different cell types. In gene expression analysis, PMA-THP-1 cells incubated with LPS had higher *TNF-α* and *IL-12* mRNA expression than THP-1 cells and also augmented *IL-10* mRNA expression. These results suggested that the responses to LPS were different in each of these cell types. In addition, LPS-A46 showed higher augmentation of *TNF-α* and *IL-10*, which indicated that the functions of LPS-A46 and LPSp were slightly different.

Next, we assessed gene expression compared to other macrophages. Two cytokines that appear to be important in controlling the number and function of macrophage lineage populations under inflammatory conditions are GM-CSF and M-CSF (CSF-1). Therefore, we prepared PBMC-derived macrophages using M-CSF and GM-CSF (M-PBDM and GM-PBDM cells). All LPSs used in this study augmented the cytokine gene expression such as *IL-12, IL-10* and *TNF-α* mRNA expression in M-PBDM cells, whereas they augmented *IL-12* and *TNF-α* mRNA expression, but not *IL-10* mRNA expression in GM-PBDM cells. Therefore, it is reasonable to suppose that the response to LPS was different for each of these phenotypes, even if these cells were derived from the same origin. In addition, LPS-A46 induced higher *TNF-α* mRNA expression than the other LPS in the macrophages, suggesting that *TNF-α* and its related molecules may be key molecules in establishing the correlations between LPS and host biological activities.

It is known that LPS can activate not only macrophages but also T- and B-cells. This study showed that LPS-A46 induced CD69 expression on $CD3^+$ cells similar to LPSe, but LPSp did not. In addition, CD69 was also induced on $CD3^-$ cells after incubation with the other types of LPS used in this study. CD69 is rapidly induced on activated T- and B-cells, neutrophils and natural killer cells (33-37). Although CD69 ligand and its function remains unclear, CD69 activation on T-lymphocytes occurs through $CD14^+$ monocytes. Therefore, our results suggest that LPS-A46 should induce different activities in monocytes and may lead to different functions from those induced by LPSp *in vivo*. CD69 may also be a key molecule for understanding the correlations between LPS and host biological activities.

In conclusion, LPS-A46 is a bioactive functional LPS that has a putatively different O-PS structure and function from that of LPS derived from symbiotic bacteria in wheat

(LPSp). Furthermore, this study showed that O-PS in LPS are involved in the expression of different macrophage phenotypes. These findings provide a basis for understanding the correlations between O-PS in LPS and host biological activities. We believe that the correlational study of the function of the characteristic LPS and polarized macrophages will be useful in the treatment and prevention of various diseases including cancer.

Acknowledgements

This study was supported by grants-in-aid for City Area Program (Developed Stage, Takamatsu area) from the Ministry of Education, Culture, Sports and Technology, Japan.

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Received April 7, 2011
Revised June 6, 2011
Accepted June 7, 2011