

## Identification and Characterization of Lipopolysaccharide in Acetic Acid Bacteria

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**Abstract.** Background: Lipopolysaccharide (LPS), a major component of the cell walls of Gram-negative bacteria, was one of the main components of Coley's vaccine and is known to have strong adjuvant activity. Though it is known that LPS exists in the digestive tract of organisms, the biological significance for the organism has not been clarified. In this study, the correlation between the structure and function of LPS was determined using acetic acid bacteria. These are Gram-negative bacteria consumed in human diets. Materials and Methods: Extracts were obtained from a strain of acetic acid bacteria which is used for producing vinegar. Determination of the LPS neutralizing activity was carried out by the Limulus test. Tumor necrosis factor (TNF) and nitric oxide (NO) production were then observed after the addition of the extracts to murine monocyte macrophages (RAW264.7), with or without Polymyxin B. TNF production in peritoneal macrophages derived from LPS-low responsive mice (C3H/HeJ) was studied after the addition of extracts. Results: The extracts were shown to be positive only in LPS-specific Limulus test and were negative in the (1,3)- $\beta$ -D-glucan-specific Limulus test. Both extracts induced NO and TNF production in RAW264.7 cells, but this was inhibited by the presence of Polymyxin B. TNF production was inhibited in peritoneal macrophages from LPS low-responsive mice (C3H/HeJ). Conclusion: LPS with

macrophage-activating activity is present in acetic acid bacteria, routinely consumed by humans.

Lipopolysaccharide (LPS) is a major component of the exterior cell walls of Gram-negative bacteria and is known to have a strong adjuvant activity; it was one of the components of Coley's vaccine (1). Intravenous administration of LPS shows strong activity in inducing the production of cytokines even at doses as low as 4 ng/kg, and causes excessive inflammation-like responses in humans which can result in endotoxin shock, hypotension or sepsis (2). As a consequence of the deleterious effects ascribable to strong cytokine induction, the toxicity of LPS, rather than its usefulness, has been the major focus of study for this substance.

Since LPS exists in various Gram-negative bacteria, including intestinal bacteria, such as *Escherichia coli* (*E. coli*) or *Enterobacter*, it is known that LPS is present in the digestive cavity of animals. However, the biological significance of LPS in the digestive tract and its effects on the host organism has not yet been studied thoroughly, although Rakoff-Nahoum *et al.* reported that signal transduction mediated by Toll-like receptor (TLR) is important for the maintenance of homeostasis of digestive tract (3) and Paltorak *et al.* demonstrated that LPS activates innate immunity through TLR4 (4). These reports suggest that the LPS in digestive tract plays an important role in the maintenance of homeostasis. Oral or percutaneous administration of LPS was reported to be effective for the therapy of various diseases including atopic dermatitis. Thus, the biological significance of LPS is beginning to be re-established (5, 6).

LPS derived from *Pantoea agglomerans* (LPSp) was found by our group to be safe after oral or percutaneous administration and showed preventive therapeutic effects

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against various diseases, including infectious diseases and cancer (7, 8). The macrophage network hypothesis in which appropriate activation of macrophages had a significant role in the maintenance of homeostasis was suggested (9) and the utility of LPS as an effective macrophage-activating substance was examined. Braun-Fahrlander *et al.* also reported that exposure to LPS was inversely proportional to the frequency of the onset of asthma in an extensive epidemiological study (5) and suggested that LPS plays an important role in the maintenance of an organism's health. These results suggest, that the macrophage network maintains homeostasis determining a correlation between the structure and function of LPS.

Our research was focused on acetic acid bacteria which have a long history of use in foods, *i.e.*, in the production of vinegar. Besides vinegar, these bacteria are used for the production of kefir yogurt and Nata De Coco, both of which are considered health foods (10, 11). Research on the oral administration of this acetic acid bacteria includes the adjuvanticity of cell wall components such as (1,4)- $\beta$ -D-glucan (12, 13). Previous research on acetic acid bacteria had focused on the identification of O-antigen (14), the presence of polysaccharides in the cellular membrane (15), or proteins that are resistant to acetic acid (16). There have been no reports on the function of LPS in acetic acid bacteria.

As the first step in analyzing the function of acetic acid bacteria, it was necessary to determine whether LPS was present in these bacteria. If LPS were present, there is a high probability that this was related to the health benefits that come from foods produced by utilizing acetic acid bacteria. In addition, determining the biological activity of acetic acid bacteria by purifying and refining LPS, may lead to an understanding of the molecular basis of the macrophage network and to the development of new, physiologically functional food products from the acetic acid bacteria and from LPS that may have beneficial effect on human health and cancer prevention.

## Materials and Methods

**Culture of acetic acid bacteria.** *Acetobacter aceti* (*A. aceti*) (NBRC No.14818<sup>T</sup>) was purchased from the National Institute of Technology and Evaluation (NITE, Japan). *A. aceti* was cultured in 75 cm<sup>2</sup> flasks with 50 ml of 804 medium (0.5% polypepton, 0.5% yeast extract, 0.5% glucose, 0.1% magnesium sulfate), at 30°C. The bacteria were subcultured every 5 days and collected by centrifuging (9,500 xg) in 500 ml tubes at 4°C, for 30 min.

Acetic acid bacteria (*Acetobacter* and *Gluconobacter*), used for actual commercial production of vinegar were provided by Tamura Zousu Corporation (Tamura's acetic acid bacteria).

**Cell lines and culture conditions.** The mouse macrophage cell line (RAW264.7) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). RAW264.7 cells were

cultured in 25 cm<sup>2</sup> flasks with RPMI-1640 (SIGMA, USA) supplemented with ampicillin (60  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml) and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and grown at 37°C under a 5% CO<sub>2</sub> atmosphere.

The mouse fibroblast-cell line, L929, was purchased from Daiichi Pharmaceutical Company (Japan) and was cultured in 75 cm<sup>2</sup> flasks with Eagle's minimum essential medium (EMEM) (SIGMA, USA) supplemented with ampicillin (60  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), 10% FBS and grown at 37°C under a 5% CO<sub>2</sub> atmosphere.

**Preparation of the acetic acid bacteria extract.** The crude extract of acetic acid bacteria was prepared from *A. aceti* and Tamura's acetic acid bacteria with the modified Westphal standard LPS purification method (17). Briefly, acetic acid bacteria were extracted with 45% phenol at 68°C for 10 min. The aqueous layer was collected after centrifugation at 12,000 xg at 4°C for 10 min. Crude extract was dialyzed with distilled water (DW) 5 to 6 times. To remove the nucleic acid, the crude extract was added to 100 units/ml DNase solution (DNase I/DNase reaction buffer (10 mM Tris-HCl buffer, pH 7.6, 2.5 mM magnesium chloride, 0.5 mM calcium chloride) at 37°C for 2 h and then to RNase (10  $\mu$ g/ml) and was incubated for 1 h at 37°C. The nuclease-treated sample was re-extracted with 45% phenol for 10 min and was centrifuged (12,000 xg) at 4°C for 10 min. The acetic acid bacteria extract was collected, dialyzed with DW, concentrated by ultrafiltration (Microcon, YM-30, 30,000MWCO, Millipore, Bedford, MA, USA) and freeze dried. The LPS content was about 60%, as calculated from the dry weight, considering the nucleic acid content estimated by absorbance at 260 nm in the solution before drying. The LPS was purified in our laboratory (17).

**Determination of LPS neutralizing activity by the chromogenic Limulus test.** The tests were performed essentially as recommended by the manufacturer with *E. coli*-LPS as the standard. Both the LPS-specific Limulus test (Endospey; Seikagaku Corporation, Japan) and the (1,3)- $\beta$ -glucan-specific Limulus test (Fungitec G; Seikagaku Corporation, Japan) were employed.

**Tumor necrosis factor (TNF) and nitric oxide (NO) production by murine macrophage.** RAW264.7 cells (8x10<sup>5</sup> cells/ml) were exposed to various concentrations of acetic acid bacteria extracts and LPSp. Six hours after exposure, the supernatant was assayed for TNF. Twenty-four hours after exposure, the supernatant was analyzed for NO release. The concentration of the LPS fraction of acetic acid bacteria was calculated based on the Endospey test. Polymyxin B was added to each culture at a final concentration of 10  $\mu$ g/ml. TNF activity was assayed by using the L929 cytotoxicity test as described previously (19). The detection limit was 3.0 units/ml. The NO concentration was measured by the Griess reaction as described previously (20, 21).

**Peritoneal macrophages from LPS low-responsive mice (C3H/HeJ) and TNF production after stimulation by LPSp and acetic acid bacteria extract.** LPS low-responsive mice (C3H/HeJ) were purchased from CLEA Japan, Inc., and were administered 2 ml of 4.05% thioglycolate broth in the abdominal cavity. After 3 days, the mice were sacrificed by cervical dislocation and were peritoneally injected with 5 ml phosphate buffered saline (PBS). The peritoneal cells were harvested with a 50-ml tube and were centrifuged at 160 xg at 4°C for 10 min. The precipitate was suspended in RPMI-1640; 1.5x10<sup>6</sup> cells/ml peritoneal

Table I. *Limulus* test of acetic acid bacteria extracts.

	<i>Acetobacter aceti</i> extract	TAMURA's acetic acid bacteria extract	LPSp
LPS-specific <i>Limulus</i> test (Endospey)	14.5 mg/g (wet)	12.5 mg/g (wet)	30 mg/g (wet)
(1→3)-β-D-glucan specific <i>Limulus</i> test (Fungitec G)	59.5 ng/g (wet)	16.6 mg/g (wet)	ND

The biological activity of lipopolysaccharide (LPS) was determined by the chromogenic *Limulus* test. The tests were performed essentially as recommended by the manufacturer as described in the Materials and Methods. LPSp = LPS derived from *Pantoea agglomerans*. ND = no detection.

macrophages were pre-cultured in 100 µl of RPMI-1640 in 96-well flat-bottomed microplates at 37°C under a 5% CO<sub>2</sub> atmosphere for 2 h, and then non-adherent cells were removed with an aspirator and adherent cells were washed with RPMI-1640. 125 µl of RPMI-1640 supplemented with ampicillin (60 µg/ml), kanamycin (50 µg/ml) and 10% FBS was added to each well. Several concentrations of acetic acid bacteria extract and LPSp were added to the peritoneal macrophages in the 96-well microplates for 6 h. The concentration of LPS in the acetic acid bacteria extracts was calculated from Endospey tests with the final concentrations of 0, 0.1, 1, 10, 30 and 100 µg/ml. LPSp at final concentrations of 0, 0.1 and 1 µg/ml and zymosan at final concentrations of 0, 0.1, 1, 10, 30 and 100 µg/ml were used. After cultivation, 50 µl supernatant from each well were transferred into other 96-well flat-bottomed microplates and subjected to biological assay.

**Statistical analysis.** Data were expressed as the mean±SD. Statistical analyses were performed using the Student's *t*-test. Differences were considered statistically significant with *p*<0.05.

## Results

***Limulus* activity in the extracts of acetic acid bacteria.** As shown in Table I, using the Endospey kit, the extract of *A. aceti* contained 12.5 mg of LPS per g wet weight of bacteria, while the Fungitec G test gave a value of 59.5 ng/g wet weight of bacteria. The Fungitec G test reacts specifically to (1,3)-β-D-glucan and fungal polysaccharide (22). The extract of Tamura's acetic acid bacteria was found to have similar values.

**Induction of TNF and NO production in RAW 264.7 cells by acetic acid bacteria extracts.** The above results suggested that both extracts of acetic acid bacteria contained LPS. However, the possibility of the presence of phospholipids could not be excluded, since it is known that factor C reacts specifically to lipid A and acidic phospholipids such as phosphatidyl inositol, phosphatidyl glycerol or cardiolipin (23). However, it was reported that phospholipids did not stimulate macrophages (24). For this reason, the biological activity of acetic acid bacteria extracts was examined by measuring the TNF and NO production by the RAW 264.7 cells. As shown in Figure 1,

more than 1 µg/ml of *A. aceti* extract induced the production of a significant amount of TNF, but this activity was almost 1,000-fold less than for LPSp. This activity was almost completely inhibited by the addition of Polymyxin B. Acetic acid bacteria extracts were observed to induce significant production of NO (Figure 2). This activity was also inhibited by the addition of Polymyxin B (Figure 2). These results support the hypothesis that the extracts of acetic acid bacteria contain significant amounts of biologically-active, *Limulus*-reaction-positive LPS.

**TNF production by C3H/HeJ macrophages.** C3H/HeJ mice are known for their LPS-hyporesponsive character, caused by a deficiency of TLR4, the major LPS receptor creating intracellular signals. Thus, LPS-like molecules in the extract of acetic acid bacteria can be estimated using the signal transduction mechanism of the macrophages. As shown in Figure 3, zymosan, which activates macrophages through TLR2, induced production of TNF, while LPSp, which activates macrophages through TLR4, did not. The extract of acetic acid bacteria did not induce TNF activity in peritoneal macrophages of C3H/HeJ mice. These results indicate that *A. aceti* extract may activate macrophages through TLR4.

## Discussion

In the present report, the extract from two types of acetic acid bacteria showed positive reactions only in the Endospey test and negative reactions in the Fungitec G test. The Endospey test contains factor C, which reacts to Lipid A, a lipidic moiety of LPS. Fungitec G is a (1,3)-β-D-glucan-specific *Limulus* kit. Factor C reacts to acidic phospholipids, such as phosphatidyl inositol, phosphatidyl glycerol or cardiolipin (23). Therefore, the positive results shown by the extracts from acetic acid bacteria in the Endospey tests suggest the presence of LPS in the bacteria; however, the possibility of the existence of phospholipids cannot be excluded. Phospholipids do not stimulate macrophages (24). Considering the results mentioned above, the Endospey-positive substance in the extract is probably LPS.

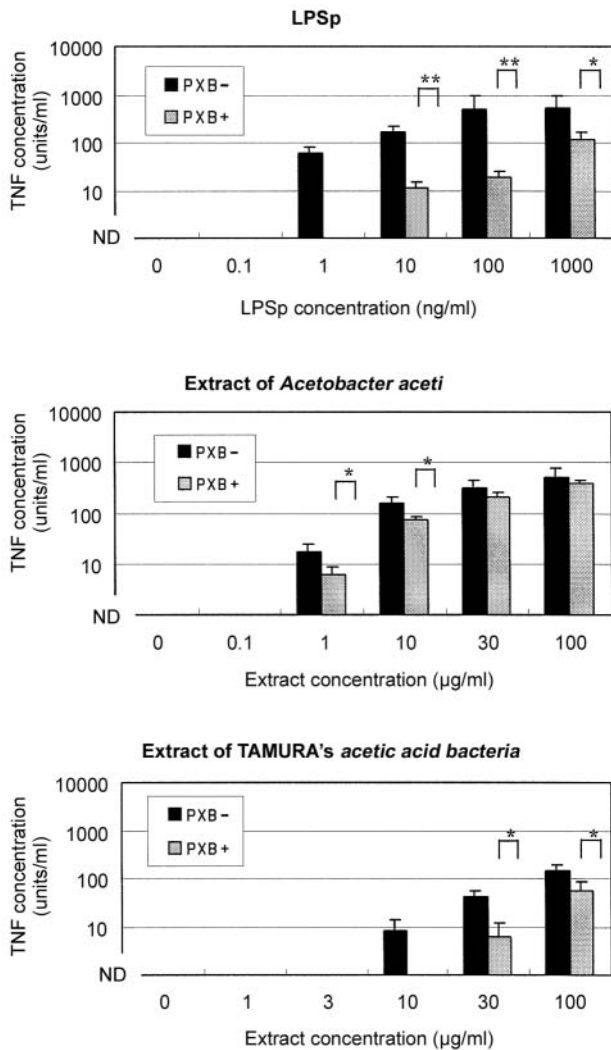


Figure 1. Tumor necrosis factor (TNF) production by RAW264.7 cells treated with lipopolysaccharide derived from *Pantoea agglomerans* (LPSp) and acetic acid bacteria extracts. After 6 h of activation, the supernatant was analyzed for TNF release. Polymyxin B (PXB) was used at a final concentration of 10 µg/ml. TNF concentrations were measured by the L929 cytotoxicity assay. The data represent means ± SD (n=3). Significant differences (\*\*p<0.01, \*p<0.05) were obtained between the groups with and without PXB. ND = detection limit below 3.0 units/ml.

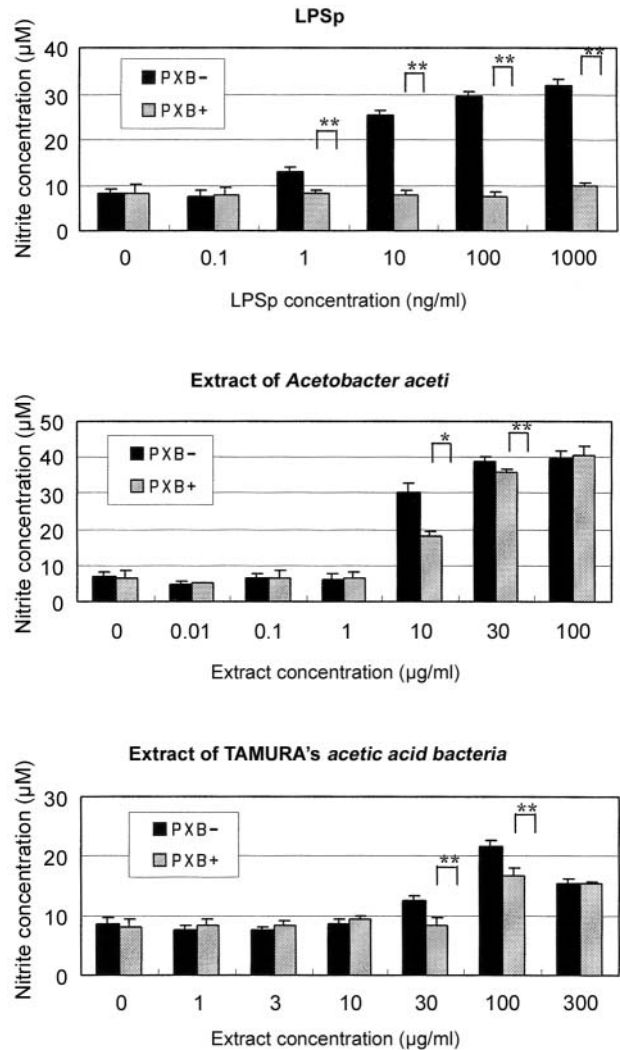


Figure 2. Nitric oxide (NO) production by macrophages stimulated by lipopolysaccharide derived from *Pantoea agglomerans* (LPSp) and acetic acid bacteria extracts. RAW264.7 cells were treated with various concentrations of acetic acid bacteria extracts and LPSp. After 24 h of activation, the supernatant was analyzed for NO release. LPSp was calculated based on the Endoscopy test. Polymyxin B (PXB) was used at a final concentration of 10 µg/ml. The concentrations of released NO in the supernatants were measured using the Griess reagent. The data represent means ± SD (n=3). Significant differences (\*\* p<0.01, \*p<0.05) were obtained between the groups with and without PXB.

As shown in Table I, the amount of LPS-like substance in the extracts from Gram-negative bacteria (as estimated by the converted concentration from the Limulus test) was low compared to that of *Pantoea agglomerans*. These results could be attributed to: i) a small amount of LPS in the bacteria; ii) a low activity to Endoscopy of the LPS-like substance in the bacteria. It has generally been found that the amount of LPS in

most Gram-negative bacteria (with the exception of *Sphingomonas* or *Fibrobacter succinogenes*, which do not contain LPS) (25), does not differ greatly. Thus, there is only a small probability that the low activity in the Endoscopy tests is caused by the small amount of LPS in the acetic acid bacteria; it seems more likely that the low activity of the LPS from acetic acid bacteria is caused by structural differences in the LPS.



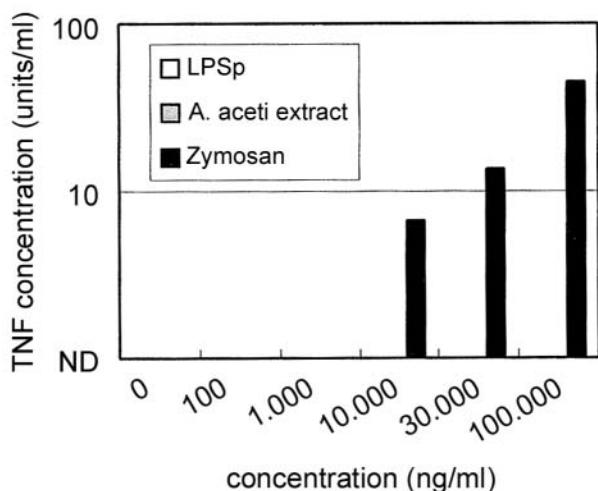


Figure 3. Tumor necrosis factor (TNF) production by C3H/HeJ peritoneal macrophages. Peritoneal macrophages of lipopolysaccharide (LPS) low responsive-mouse (C3H/HeJ) were treated with various concentrations of *Acetobacter aceti* extract, Zymosan extract and LPS derived from *Pantoea agglomerans* (LPSp). After 6 h of activation, the supernatant was assayed for TNF activity with the L929 cytotoxicity assay. ND = detection limit below 3.0 units/ml.

As shown in Figures 1 and 2, the extracts from acetic acid bacteria induced production of NO and TNF by RAW264.7 cells in the same manner as LPSp, suggesting the presence of Lipid A in the extract and the possibility that the Endospey-positive substance in the bacteria is LPS. Although the LPS from acetic acid bacteria was found to activate macrophage in the same manner as LPSp, their specific activity to induce macrophage activation was different (as compared to the value calculated from the activity in the Limulus tests) (Table I and Figures 1, 2). The specific activity for macrophages activation was reported to differ greatly depending on the structure of Lipid A (26). Thus, the effective level of activation is believed to be a reflection of structural differences of the LPS. It was also reported that the effective level that activates macrophages differs depending on the structure of sugar chain (27). However, details concerning the sugar content of acetic acid bacteria are not yet known.

In the experiments on the signal transduction mechanism of the LPS present in acetic acid bacteria, induction of TNF production by the extract was not observed in peritoneal macrophages of LPS low-responsive mice (Figure 3). NO production showed a similar tendency (data not shown). Therefore, the extract from acetic acid bacteria is believed to activate macrophages through TLR4 in the same manner as LPSp.

To summarize, a substance in acetic acid bacteria has the following four characteristics: i) positive for the

Endospey test; ii) macrophage-activating activity; iii) inhibition of its activity by the addition of Polymyxin B; and iv) TLR4 mediated signal transduction of macrophage activation. The only substance that is known to satisfy all these conditions is LPS. However, the structure of LPS from the acetic acid bacteria appears to be different from that of LPSp. It is currently unknown whether this structural difference is due to Lipid A or to the sugar moiety. When these facts are understood, it should be possible to explain the biological activity of LPS derived from acetic acid bacteria.

The route of LPS-absorption may clarify the differences between the LPS of bacterial flora in the intestine and the mechanism of action underlying the oral administration of LPS with food products. The macrophage network, involved in the cellular integrity of the body, maintains homeostasis receiving external environmental information and transmitting it within the organism. Therefore, the LPS from acetic acid bacteria, which cause various quantitative and qualitative effects on macrophage activation may also have different effects on health maintenance than LPSp. Thus, new physiologically functional foods that incorporate these attributes could be developed.

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