Biological Effects of Lipopolysaccharide from *Achromobacter stenoalis* on Lymphocytes and Macrophages

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**ABSTRACT.** The immunopotentiating activities of lipopolysaccharide from *Achromobacter stenoalis* (A-LPS) were examined. A-LPS was structurally atypical and gave no endotoxin shock in A-LPS-inoculated mice. Analysis in vitro showed that A-LPS was a potent activator of both macrophages and B-lymphocytes. After macrophage stimulation with A-LPS, interleukin-1 (IL-1) secretion, interferon (IFN) production and chemiluminescence (CL) response were induced. A-LPS was a potent mitogen for spleen lymphocytes. However, induction of interleukin-2 (IL-2) secretion in T lymphocytes was not observed. These activities of A-LPS were similar to or higher than that of enterobacterial LPS. —**KEY WORDS:** *Achromobacter stenoalis*, biological effect, lipopolysaccharide, lymphocyte, macrophage.


An impressive array of biological activities is attributed to the lipopolysaccharide (LPS) component of the cell wall of gram-negative bacteria. A partial list of these activities would include the capacity of LPS to act as a B lymphocyte mitogen [3], a T-independent antigen [16], a polyclonal B-lymphocyte activator [2], an adjuvant [9], macrophage activator [17], a lethal toxin [18], an inhibitor tolerance induction [14], an activator of serum component and others [17]. The chemical composition and structure of some classical LPS have been studied in detail by a number of investigators and various types of lipid A have been synthesized [4, 20]. The different types of LPS have been extracted from bacteria such as *Bacteroides*, *Leptospira* and others [11, 12, 13]. The biological properties of them were not equal to that of classical LPS. Some LPSs have been demonstrated to be more effective activator in immunomodulation. Therefore, LPS can alter susceptibility to infection and have provided an important tool for investigation of the mechanisms of host resistance.

Recent studies indicated that many of immunomodulating agents were effective in disease (e.g., tumor)-affected animals and man [15]. Both preclinical and clinical studies are presented describing the therapeutic response attained with various biological response modifying agents and specific effector cells regulated by these agents. Research for newly production or discovery of the agents has been continued.

*A. stenoalis* is a gram-negative and aerobic organism which has been isolated from sea water. The anti-tumor effect in the heat killed organisms was observed in tumor bearing mice [8]. The active components, however, have been not identified.

In the research reported here, A-LPS was extracted from the organisms and was
observed to be the potent activities for immunomodulation. The biological effects on lymphocytes and macrophages will be discussed.

**MATERIALS AND METHODS**

**Culture of the bacteria:** *Achromobacter stenohalis* (marine *Alkarigenes* spp.) strain Hongkong [7] was used. The bacteria were aerobically grown at 30°C on Marine agar 2216 (Difco) for 24 h.

**Isolation and purification of A-LPS:** Harvested cells were washed three times with sterile distilled water, freeze-dried, and subjected to hot phenol-water extraction. The crude A-LPS extract was further purified with RNAase and ultracentrifugation repeated 3 times (100,000 × g, 3h). The purified A-LPS was lyophilized and stored at −80°C.

Cell wall fraction was obtained by the method of Syuto et al. [19] and used for comparative studies. *Escherichia coli* LPS (E-LPS) from *E. coli* 0111 B4 (Difco), and *Salmonella minnesota* LPS (S-LPS) from *S. minnesota* R595 (Difco) were also used for comparative studies.

**Chemical analysis:** Chemical analysis of A-LPS was done according to the method previously described [12]. For analysis of neutral sugars, a sample of A-LPS was hydrolyzed in 1N-HCl at 70°C for 30 min. The sugar contents were derivatized and analyzed by high-performance liquid chromatography (HPLC, LC-6A, Shimazu Co.) using an SCR-101N column (Shimazu Co.).

**Injection of A-LPS:** A total of 120 ddY mice (male, 6-week-old) was used for LD₅₀ assay and histopathological examinations. The mice were divided into 3 groups receiving intravenous injection (*iv*) of A-LPS, *iv* injection of E-LPS and no injection, respectively. Various concentrations (31.3, 62.5, 125, 250, 500 and 1000 µg/mouse) of LPS were injected into each mouse of the *iv* groups. Mice were sacrificed at 1, 3, and 7 days after LPS injection. The liver, lung, spleen and other organs of the mice were examined for histopathologically. Tissues were collected, fixed in 10% formalin, embedded in paraffin, sectioned and stained by hematoxyline and eosin.

**Induction of interleukin-1 (IL-1) secretion:** BALB/c mouse peritoneal macrophages were prepared by intraperitoneal injection of 5% proteose peptone and 5% starch. After 72 h, peritoneal exudate cells were collected by peritoneal lavage with 5 ml of Hank’s balanced salt solution (HBSS, Gibco), washed and resuspended in RPMI 1640 medium. The peritoneal cells (2.4 × 10⁶ cells) were plated in 35 mm Petri dishes and incubated at 37°C in 5% CO₂ for 2 h and washed 3 times with HBSS containing 1% FBS. The adherent cells were incubated in RPMI 1640 medium containing 10% FBS, with or without stimulants, for 24 h. Supernatants were collected and cellular components were removed by means of a Millipore filter.

IL-1 activity in culture supernatants was determined as described by Vacheron et al. [21]. In brief, thymocytes from BALB/c mice were suspended to a density of 7 × 10⁶ cells per ml in RPMI 1640 medium containing antibiotics, 10% FBS and 20mM Hepes. The cell suspension (0.1 ml) was seeded into a 96-well microculture plate, and an equal volume of a 1:4 dilution of macrophage culture supernatants was added. Cultivation was carried out with or without the addition of submitogenic concentration of Con A (1 µg/ml) and incubated in a 5% CO₂ atmosphere for 48 h. Cultures were pulsed for the final 6 h with 0.25 µCi of ³H thymidine ([³H]-TdR, sp. act. 2.0 Ci/mmol). The cells were harvested on glass fiber filters and radioactivity was measured by means of a liquid scintillation counter (Aloca, Japan).

**Interferon (IFN) assay:** The IFN assay was performed by a semimicro, dye-binding assay method [5], based on quantification of
the inhibition of the cytotoxic effect in the microplate. The peripheral blood mononuclear leukocytes (PBML) were obtained from normal human donors using the Ficoll-Hypaque gradient centrifugation technique. The supernatant of the PBML culture (1 × 10^6 cells) was used for the IFN assay after 3 days of incubation with LPS. Culture of human amion cells (FL) were exposed to serially twofold diluted samples, challenged with vesicular stomatitis virus (New Jersey strain, 10^6 PFU/ml), and stained with gentian violet. The bound dye was then eluted and measured colorimetrically. A laboratory standard which had been calculated from the NIH standard (G-023-901-527) was inoculated into each microplate. The IFN titer of the samples was calibrated with the aid of the standard and expressed as international IFN units (IU)/ml.

Chemiluminescence analysis: Chemical analysis was done according the method previously reported [1, 12]. C3H/HeJ mice 8 to 10 weeks of age were used as source of peritoneal macrophages which had been elicited by i.p. injection of 5% proteose peptone containing 5% starch. Peritoneal exudate cells were washed 3 times with RPMI 1640 medium and suspended (5 × 10^5 cells/ml) in the medium supplemented by 10% heat-inactivated FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) and Hepes (20 mM). One hundred µl of macrophage suspension and 10 µl of luminol solution (2 mg/ml) were mixed. After 10 min, 10 µl of A-LPS (final concentration 0.2, 2.0 and 20.0 µg/ml) was added. Then the chemiluminescence response (CLR) was measured by a luminescence analyzer (Biolomat LB9500). E-LPS was also used for negative control.

Mitogenic activity: A spleen cell suspension (10^7 cells/ml) in RPMI 1640, supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and heat-inactivated fetal bovine serum (FBS, GIBCO) was prepared from BALB/c mice. One hundred µl of LPS (50 µg/ml, A-LPS, E-LPS or S-LPS) was added to each well. Incubation was done in 5% CO₂ for 48 h at 37°C. [³H] thymidine (0.25 µCi/well, [³H]TdR, sp. act. 2.0 Ci/mmol, Amersham Int.) was added to each well 6 h before incubation. Cells were harvested and levels of radioactivity were measured by a liquid scintillation counter (Aloca, Japan).

IL-2 assay: IL-2 activity was measured by the method of previous report [10]. Splenic lymphocytes (10^8 cells/ml) from BALB/c mice were prepared. Macrophages were eliminated from the preparation by the incubation with carbonyl iron (0.2 g/10^8 cells, 37°C 1 h incubation, Wako Chemical Co.), by method of Ficoll-Conray centrifugation and next by incubation in Petri dish. Splenic lymphocytes without monocytes/macrophages (1 × 10^6 cells/ml) were cultured with A-LPS (final concentration of 10 µg/ml) for 48 h at 37°C in a 5% CO₂-humidified atmosphere. Supernatants were collected and cellular components were removed by means of a Millipore filter.

IL-2 activity in culture supernatants was assayed using an IL-2 dependent cloned murine cytotoxic T cell line (CTLL-2). CTLL-2 cells were washed 3 times and then seeded at 100 µl (1 × 10^5 cells/ml) in each well of a 96-well flat bottom microtiter plate. The supernatants (100 µl, various dilution) were added to each well. The cells were pulsed with 0.25 µCi of [³H]TdR (sp. act. 2.0 Ci/mmol, Amersham Int.) 24 hr before terminating the cultures. Cells were then harvested onto glass fiber filters with a multiple-sample harvester. The amount of incorporated radioactivity was measured as IL-2 activity.

RESULTS

Chemical properties of A-LPS: The final
product contained 21.6% carbohydrate, 13.3% lipid and 9.4% protein. Heptose and 2-keto-3-deoxyoctonate (KDO) were not detected. The 5 main sugars were detected in HPLC analysis (Fig. 1). They, however, showed different retention time with comparison of standard sugars and were not identified.

Toxicity of A-LPS: After injection of A-LPS into mice, death was observed within 1 h (mainly within 20 min) at sufficient high dosage. These mice, however, did not show any clinical signs of endotoxin shock and histological lesions. In mice inoculated with E-LPS, in contrast, death was observed with endotoxin shock. ED$_{50}$ of A-LPS was 210 µg/mouse, compared to 270 µg/mouse for E-LPS.

A-LPS showed no effect of body weight on the survived mice (Fig. 2). In contrast, body weight was decreased and not recovered in mice inoculated with E-LPS.

Histological changes in mice after iv injection of A-LPS: The injection of A-LPS induced histological changes in the liver and spleen of mice. In survived mice, activation and increasing number of macrophages and lymphocytes were observed. The organ cell damage was very mild or none in the mice. In died mice, congestion of various organs was seen but any characterized lesions were not determined. In contrast, E-LPS induced multifocal necrosis, hemorrhages, congestion and disseminated intravascular coagulation in both survived and died mice.

Induction of IL-1 secretion from macrophages: IL-1 activity was assessed by measuring the incorporation of $[^3]$H]TdR by cultured murine thymocytes. The results are shown in Table 1. The culture supernatant of the macrophage which had been stimulated with A-LPS showed IL-1 activity against the mouse thymocytes. Similar activity was detected in A. stenohalis cell wall fraction and E-LPS.

Interferon production by A-LPS: The PBML preparations obtained from normal human donors produced IFN in culture fluid with stimulation of A-LPS (Table 2). IFN production observed in PBML from donor A, B, C, E, F and not in that from donor G. IFN production was enhanced with increasing of the cell numbers of PBML (data not

![Fig. 1. HPLC pattern of A-LPS after hydrolysation with HCl. Separation on SCR-101N, using H$_2$O as mobile phase with detection by FLD-6A. A: A-LPS. B: Standard, 1: maltotriose, 2: sucrose, 3: raffinose, 4: galactose, 5: fructose, 6: glucose.](image)

![Fig. 2. LPS-induced weight loss in mice.](image)

$\Delta$---$\Delta$: A-LPS, 62.5 µg/mouse; $\square$---$\square$: A-LPS, 125.0 µg/mouse; $\bigcirc$---$\bigcirc$: A-LPS, >250 µg/mouse; $\blacktriangle$---$\blacktriangle$: E-LPS, 125 µg/mouse; ●●: Control without LPS.
Table 1. Induction of IL-1 secretion by LPS in BALB/c mice

<table>
<thead>
<tr>
<th>Stimulant added to macrophages</th>
<th>[3H] TdR uptake (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-LPS</td>
<td>746±86</td>
</tr>
<tr>
<td>A. stenohalis cell wall</td>
<td>756±79</td>
</tr>
<tr>
<td>E-LPS</td>
<td>719±71</td>
</tr>
<tr>
<td>NONE</td>
<td>272±20</td>
</tr>
</tbody>
</table>

a) Thymocytes from BALB/c mice were suspended to a density of 7×10^6 cells per ml in RPMI 1640 medium. The cell suspension (0.1 ml) and an equal volume of 1:4 dilution of supernatants of macrophage cultures that had been stimulated with stimulants were seeded into a 96-well microculture plate. Cultivation was carried in the presence of Con A (0.2 μg). Data were expressed as the mean ±SE of three different cultures.

Table 2. IFN production of PBML from various donors by A-LPS

<table>
<thead>
<tr>
<th>Concentration of A-LPS (μg/ml)</th>
<th>IFN (IU/ml) from donor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>1.0</td>
<td>16</td>
</tr>
</tbody>
</table>

a) -: Not done.

Table 3. IFN production of adherent and non-adherent cell fraction by A-LPS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IFN (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PBML)</td>
<td>J</td>
</tr>
<tr>
<td>Whole</td>
<td>24</td>
</tr>
<tr>
<td>Adherent</td>
<td>12</td>
</tr>
<tr>
<td>Non-adherent</td>
<td>4</td>
</tr>
</tbody>
</table>

a) The number of cell was 1×10^5/well.

Table 4. Acid-stability of IFN samples

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>16</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>12</td>
</tr>
</tbody>
</table>

a) Samples were dialyzed against 0.2 M KCl-HCl buffer, pH 2.0, at 4°C for 24 h. Control samples were dialyzed against PBS of pH 7.4 instead of pH 2.0.

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shown). Induction of IFN production was not detectable after stimulation with S-LPS.

IFN production was observed mainly in adherent cell fraction, while little in non-adherent cell fraction (Table 3). The IFN titer was constant after treatment of pH 3.0 (Table 4). Therefore, the IFN was considered to be IFNγ/β but not IFNγ.

Chemiluminescence response (CLR)

A-LPS induced chemiluminescence response of macrophages from C3H/HeJ mice. Fig. 3 shows typical responses. Table 5 shows the counts and peak times of CLR of the macrophages in the presence of A-LPS. Dose-dependent increase of the counts was observed after stimulation with A-LPS. E-LPS did not induce CLR, because C3H/HeJ mice were non-responder to classical LPS.

Mitogenicity of A-LPS

Table 6 shows the mitogenic responses of spleen cells to LPSs. A higher mitogenic response was found in BALB/c mouse spleen cells stimulated with A-LPS than in those stimulated with E-LPS and S-LPS.

Induction of IL-2 secretion

A-LPS did not induce IL-2 secretion from spleen lymphocytes. The culture supernatant of the spleen cells which had been
stimulated with A-LPS was not significantly different from that of the non-stimulated cells.

**DISCUSSION**

The results lend strong support to the notion that a major site of action of A-LPS is the phagocytic cell system, namely the monocyte/macrophage system. Macrophage activation by A-LPS results in release of several mediators such as IL-1 and IFNα/β. As a result of interaction with A-LPS, the phagocytic cells become activated and appear to be characterized by increased microbicidal activities such as oxygenation. Activation of the phagocytic cell system is probably an important mechanism by which A-LPS is able to stimulate non-specific resistance against microbial infection.

The treatment of infections with immunostimulating drugs is broadly termed a "prohost" approach since the therapy is designed to increase host defense rather than kill specific pathogen. In recent years, many experimental works support the concept that nonspecific immunostimulants have usefulness in both the prevention and treatment of infectious diseases [6, 15].

We showed that A-LPS possesses many of beneficial properties and little of the harmful attributes of the endotoxic molecule. It has not been possible to utilize endotoxin materials in clinical applications, because of the extremely high susceptible of humans to the toxic nature of endotoxins. A-LPS did not induce endotoxin shock and pathological changes in the LPS inoculated mice. Survived mice showed increasing of body weight similar to control mice. Attenuating the endotoxin's toxic nature without eliminating its ability to stimulate beneficial immune responses was found to be possibly by removal of the phosphate moiety at the C-1 position of the toxic diphosphoryl lipid A molecule [20]. In future, the structure of A-LPS will be clarified and the activity of lipid molecule will be discussed with comparison of the synthetic adjuvants.

With relatively few exceptions, the molecule of classical LPS has been found to

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**Table 5.** Chemiluminescence response of macrophages from C3H/HeJ mouse after stimulation with A-LPS

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Concentration of stimulant</th>
<th>Max peak counts</th>
<th>Peak Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-LPS</td>
<td>20.0</td>
<td>2.2×10^5</td>
<td>29</td>
</tr>
<tr>
<td>A-LPS</td>
<td>2.0</td>
<td>1.8×10^5</td>
<td>30</td>
</tr>
<tr>
<td>A-LPS</td>
<td>0.2</td>
<td>4.5×10^5</td>
<td>29</td>
</tr>
<tr>
<td>E-LPS</td>
<td>2.0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a) =: Not detected.

**Table 6.** Mitogenic responses^3) of BALB/c mouse spleen cells to LPS

<table>
<thead>
<tr>
<th>Stimulants</th>
<th>[H] uptake (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-LPS</td>
<td>9359±744^9</td>
</tr>
<tr>
<td>E-LPS</td>
<td>6456±875</td>
</tr>
<tr>
<td>S-LPS</td>
<td>4447±935</td>
</tr>
<tr>
<td>NONE</td>
<td>605±106</td>
</tr>
</tbody>
</table>

a) BALB/c mouse spleen cells (7×10^5 cells) were incubated in a total volume of 0.2 ml of RPMI 1640 medium with LPSs (5 μg) for 48 hours.

b) Data were expressed as the mean ± SD of three different cultures.
consist of three integrated components [4, 17]: a somatic or surface antigen composed of an array of sugars, and a lipid A region apparently buried in the hydrophobic portion of the outer membrane. The lipid A consists of a glucosamine phosphate residue with bound fatty acids. The third component uniting these two units by covalent bonds is an inner core of KDO and heptose. In most gram-negative bacteria the amount of carbohydrate and lipid A falls in characteristic ranges. In some bacteria, however, there is a large variation in the distribution of KDO and heptose [11, 12, 13]. KDO is not detected in A-LPS in the present study. KDO negative LPS has been reported in the Bacteroides and Leptospira species. In recent studies, Bacteroides and Leptospira species were shown to have the different type of KDO, i.e., phospharyl KDO [7]. It may be that A-LPS also has different type of KDO. The non-detectable or low concentration of KDO and heptose may predispose it to a greater (i.e., more potent) reactivity for its host. It is possible that different structure, linkage and modification of the components of LPS molecule may be correlated with low endotoxicity.

REFERENCES

Achromobacter stenohalis 由来リポ多糖のリンパ球およびマクロファージに対する種々の生物活性：


要約

Achromobacter stenohalis 由来リポ多糖のリンパ球およびマクロファージに対する種々の生物活性：

 salir uzes 1) 佐藤昭子, 2) 藤井順弘, 3) 小熊恵二, 4) 張 国利, 5) 出口英三郎, 6) 高野一雄, 7) 正信, 8) 越岡茂郎 (東日本学園大学歯学部口腔解剖学教室), 9) 東京歯科大学歯学部口腔衛生学教室, 10) 日本製薬株式会社, 11) 鹿児島大学薬学部附属医療病院, 12) 北海道大学獣医学部実験動物学教室) —— Achromobacter stenohalis 由来のリポ多糖 (A-LPS) の生物活性を調べた。A-LPS は典型的なリポ多糖と構造が異なっていた。A-LPS を投与されたマウスはエンドトキシンショックを起こさなかった。A-LPS は in vitro においてマウスおよびヒトのマクロファージおよび B リンパ球に対し強い活性を示した。すなわち A-LPS でマクロファージを刺激したところ、IL-1 分泌、IFN の産生および化学発光が誘導された。A-LPS は脾臓 B リンパ球に対し強いマイトジェン活性を有していた。しかし、T リンパ球における IL-2 分泌の誘導は観察されなかった。A-LPS の IL-2 以外のこれらの活性は腸内細菌由来の LPS よりも高かった。