Immunobiological Properties of Lipopolysaccharides Isolated from *Fusobacterium nucleatum* and *F. necrophorum*

By NOBUO OKAHASHI, TOSHIHIKO KOGA, TATSUJI NISHIHARA, TAKU FUJIWARA AND SHIGEYUKI HAMADA*†

Department of Dental Research, The National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Lipopolysaccharides (LPSs) were isolated from *Fusobacterium nucleatum* ATCC 10953 and *F. necrophorum* ATCC 25286 by the hot phenol/water procedure. *F. nucleatum* LPS was composed of 16% (w/w) carbohydrate, 10% (w/w) hexosamine and 40% (w/w) fatty acid, while *F. necrophorum* LPS was composed of 26% (w/w) carbohydrate, 12% (w/w) hexosamine and 28% (w/w) fatty acid. These LPS preparations induced mitogenic responses in spleen cells of BALB/c, BALB/c (nu/nu) and C3H/HeN mice, and these responses were suppressed by the addition of polymyxin B. The preparations also induced the polyclonal responses of C3H/HeN spleen cells. In addition, enhanced glucose utilization and interleukin-1 production by murine peritoneal macrophages were demonstrated. Neither spleen cells nor macrophages from the ‘LPS-nonresponsive’ C3H/HeJ mouse were activated by LPSs from the *Fusobacterium* species.

**INTRODUCTION**

*Fusobacterium nucleatum* and *F. necrophorum* are Gram-negative, obligatorily anaerobic filamentous bacteria. *F. nucleatum* is prominent in the bacterial flora of dental and subgingival plaques (Slots, 1979; Socransky et al., 1982), with higher numbers than normal commonly found in sites with gingivitis or periodontitis (Moore et al., 1982; Savitt & Socransky, 1984). It has induced periodontitis in monoinfected gnotobiotic rats (Irving et al., 1978). *F. necrophorum* is rarely isolated from oral flora; however, it occasionally causes severe necrotic infections in man and animals (Duerrden, 1983).

The pathogenicity of these *Fusobacterium* species may be related in part to their endotoxin (lipopolysaccharide; LPS) (Hofstad, 1982). It has been shown that the amount of endotoxin in gingival and periodontal tissues correlates with the severity of inflammation in these sites (Shapiro et al., 1972). Simon et al. (1972) also reported that the number of anaerobic Gram-negative rods in gingival exudates correlates with the amount of endotoxin in crevicular fluid as well as with the degree of tissue inflammation. Endotoxic biological properties of various LPS preparations isolated from *Fusobacterium* species have been studied by several workers. LPS from *F. nucleatum* produces local and generalized Shwartzman reactions, and it is lethal to mice and chick embryos, and pyrogenic in rabbits (Mashimo et al., 1985; Sveen, 1977; Sveen et al., 1977). It can also activate human and guinea-pig complements (Hawley & Falkler, 1978; Nygren et al., 1979), and stimulate bone resorption (Sveen & Skaug, 1980). LPS from *F. necrophorum* also possesses various endotoxic activities (Garcia et al., 1975; Warner et al., 1975). However, little is known about the immunobiological potencies of *Fusobacterium* LPS with

† Present address: Department of Oral Microbiology, Osaka University Faculty of Dentistry, 1-8 Yamadaoka, Suita, Osaka, 565 Japan.

Abbreviations: Con A, concanavalin A; FCS, foetal calf serum; IL-1, interleukin-1; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonic acid); PFC, plaque-forming cell(s); SRBC, sheep red blood cell(s).

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respect to lymphoid cells and macrophages. In this study, we isolated and characterized LPSs from *F. nucleatum* and *F. necrophorum* by the hot phenol/water procedure, and investigated their immunobiological properties.

**METHODS**

Bacterial strains and growth conditions. *F. nucleatum* ATCC 10953 was kindly supplied by Dr T. Umemoto, Department of Oral Bacteriology, Kanagawa Dental College, Yokosuka, Japan. *F. necrophorum* ATCC 25286 was obtained from the American Type Culture Collection, Rockville, Md, USA. The bacteria were grown in Brain Heart Infusion broth (Difco) supplemented with 0.5% (w/v) yeast extract (Difco) and cysteine. HCl (0.3 mg ml⁻¹) at 37 °C for 3 days in air/CO₂ (95:5, v/v). Organisms were harvested by centrifugation at 6000 g for 30 min, washed three times with pyrogen-free water, and lyophilized.

Extraction and purification of LPS. LPS was isolated from lyophilized cells by the hot phenol/water extraction procedure of Westphal & Jann (1965). The crude extract was treated with nuclease, washed extensively with pyrogen-free water by ultracentrifugation, and lyophilized (Koga et al., 1985). This preparation was referred to as 'partially purified' LPS. The partially purified preparation was dissolved in pyrogen-free water at a concentration of 2 mg ml⁻¹, and an equal volume of 90% (v/v) phenol was added. The mixture was stirred vigorously at 65 °C for 30 min, and the aqueous phase was then collected. This extraction procedure was repeated once more. The combined aqueous phases were dialysed extensively against distilled water, lyophilized, and the product termed 'purified' LPS. LPS from *Escherichia coli* K235 was isolated and purified as described previously (Koga et al., 1985).

Chemical analyses. Hexose, hexosamine, heptose, 2-keto-3-deoxyoctonate (KDO), protein, fatty acid esters, and phosphorus were assayed by the colorimetric methods previously described (Koga et al., 1985). For sugar and fatty acid analyses, LPS was hydrolysed for 6 h at 100 °C in 2 M-HCl. The hydrolysate was suspended in chloroform/methanol/water (4:10:5, by vol.) and mixed vigorously. The aqueous and chloroform phases were separated by centrifugation, and were processed for the sugar and fatty acid analyses, respectively. Neutral and amino sugars in the aqueous phase were identified as trimethylsilyl or alditol acetate derivatives by gas–liquid chromatography (GLC), and fatty acids in the chloroform phase were quantified as their methyl esters by GLC (Koga et al., 1985).

Mitogenic and polyclonal B cell activation activities. Mitogenic activity of LPS for spleen cells from BALB/c, BALB/c (nu/nu), C3H/HeN and C3H/HeJ mice, and for thymocytes from BALB/c mice, was determined as previously described (Koga et al., 1985). Spleen cells (5 x 10⁵) were suspended in 0.2 ml RPMI 1640 medium supplemented with penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and HEPES buffer (15 mm, pH 7.2) (hereafter, this medium is referred to simply as RPMI 1640 medium), and were cultured with various stimulants at 37 °C in a humidified atmosphere of air/CO₂ (95:5, v/v). Cultures were pulsed for the final 6 h of incubation with [³H]thymidine (0.25 μCi, 9.25 kBq; Amersham), and then the amount of [³H]thymidine uptake was determined. Concanavalin A (Con A; Sigma) was used as a T-cell mitogen. The effect of polymyxin B (Sigma) on mitogenic responses of BALB/c spleen cells to LPS was examined as follows. Spleen cells (5 x 10⁵) were cultured in 0.2 ml RPMI 1640 medium containing various amounts of polymyxin B (0–5 μg) and 5 μg of stimulant. The mitogenic responses were determined as described above.

In vitro polyclonal B cell activation activity of LPS for C3H/HeN and C3H/HeJ spleen cells was determined as previously described (Koga et al., 1985). After incubation for 4 days at 37 °C in air/CO₂ (95:5, v/v), cells were removed from each culture well, washed, and assayed for direct anti-sheep erythrocyte plaque-forming cell (anti-SRBC PFC) responses by the haemolytic plaque technique of Cunningham & Szenberg (1968).

Glucose consumption by peritoneal macrophages. Macrophage activation was evaluated by the glucose consumption assay described by Ryan et al. (1979). Murine peritoneal macrophages were induced by intraperitoneal injection of 2 ml 10% (w/v) Trypticase peptone (BBL). Four days later, peritoneal exudate cells were collected by peritoneal lavage with 5 ml Hank's balanced salt solution (GIBCO) containing 1% (v/v) foetal calf serum (FCS; Armour Pharmaceutical Co.), washed, and resuspended in RPMI 1640 medium containing 5% (v/v) FCS. Peritoneal macrophages (6 x 10⁵) were placed in 96-well microtitre plates and incubated for 3 h at 37 °C in air/CO₂ (95:5, v/v), then nonadherent cells were removed by washing with RPMI 1640 medium. The adherent cells were cultured in 0.2 ml RPMI 1640 medium with or without stimulant at 37 °C in air/CO₂ (95:5, v/v), and samples (5 μl) of the culture supernatant were assayed for glucose using a blood sugar GOD-Perid test kit (Boehringer Mannheim). The results were expressed as percentages of the initial glucose concentration in the culture supernatants. Each assay was done on triplicate cultures.

Induction of interleukin-1 (IL-1) production by peritoneal macrophages. Peritoneal macrophages (3 x 10⁵) prepared as described above were cultured in 1 ml RPMI 1640 with or without stimulant at 37 °C in air/CO₂ (95:5, v/v) for 24 h. The culture supernatant was harvested by centrifugation, and passed through a filter membrane (0.45 μm pore size, Millipore). IL-1 activity in the culture supernatant was quantified by measuring the incorporation of [³H]thymidine into C3H/HeJ mouse thymocytes (Vacheron et al., 1983). The cells (1·5 x 10⁶) were cultured for
**RESULTS**

Chemical composition of LPSs from *Fusobacterium* spp.

Purified LPS from *F. nucleatum* ATCC 10953 (*F. nucleatum* LPS) contained (w/w) 15.9% carbohydrate, 10.4% amino sugar, 40.0% fatty acid and 4.9% phosphorus. Purified LPS from *F. necrophorum* ATCC 25286 (*F. necrophorum* LPS) contained 26.1% carbohydrate, 11.5% amino sugar, 27.9% fatty acid and 3.1% phosphorus. These LPS preparations contained less than 2% protein as determined by the Lowry procedure. The major sugars in *F. nucleatum* LPS were galactose and heptose, whereas those in *F. necrophorum* LPS were glucose and heptose. The major fatty acids in *F. nucleatum* LPS were myristic (C14:0), P-hydroxymyristic (3-OH C14:0) and β-hydroxypalmitic (3-OH C16:0) acids. *F. necrophorum* LPS did not contain β-hydroxypalmitic acid (data not shown).

Mitogenic and polyclonal B-cell activation activities of LPS

Purified LPSs from *F. nucleatum* and *F. necrophorum* were mitogenic for spleen cells of BALB/c and BALB/c (nu/nu) mice, but not for BALB/c thymocytes (Table 1). Similar activities were also found with the reference *E. coli* LPS. These results indicate that LPSs from *Fusobacterium* species, like *E. coli* LPS, are B-cell mitogens. Purified LPS preparations from *F. nucleatum* and *F. necrophorum* were clearly mitogenic for spleen cells of C3H/HeN mice but not for those of C3H/HeJ mice (Fig. 1a, b). However, 'partially purified' LPSs from both species were significantly mitogenic for C3H/HeN spleen cells (Fig. 1c, d). This may have been due to contaminating endotoxic proteins, since these 'partially purified' LPS preparations contained significant amounts of protein (5.9% and 4.3% for *F. nucleatum* and *F. necrophorum*, respectively).

We examined the effect of polymyxin B, which inhibits mitogenic activity of *Enterobacteriaceae* LPS, on the mitogenicity of LPSs from the *Fusobacterium* species (Fig. 2). Polymyxin B markedly suppressed the mitogenic activity of purified LPSs from *Fusobacterium* species as well as that of LPS from *E. coli*, but it only weakly inhibited the mitogenicity of Con A, used as a reference T-cell mitogen.

Purified LPSs from both *F. nucleatum* and *F. necrophorum* enhanced *in vitro* direct PFC responses of C3H/HeN spleen cells to SRBC, but not those of C3H/HeJ spleen cells (Table 2). These results suggest that the LPS preparations induced polyclonal IgM synthesis in C3H/HeN but not in C3H/HeJ mice. Both LPS preparations showed *in vitro* adjuvant activity for C3H/HeN spleen cells, but not for C3H/HeJ spleen cells (data not shown).

Activation of macrophages by LPS

The ability of the purified LPSs to enhance the metabolic rate of macrophages was evaluated by measuring glucose consumption. Both *F. nucleatum* and *F. necrophorum* LPSs enhanced glucose utilization by BALB/c peritoneal macrophages, to the same extent as *E. coli* LPS (Fig. 3). C3H/HeJ macrophages were not stimulated by these LPS preparations (Fig. 4).

IL-1 activity in the culture supernatant of peritoneal macrophages was assessed by measuring the incorporation of [3H]thymidine by C3H/HeJ thymocytes. Both *F. nucleatum* and *F. necrophorum* LPSs stimulated C3H/HeN macrophages but not C3H/HeJ macrophages, to produce IL-1 activity (Table 3).

DISCUSSION

Chemical analyses revealed that *F. nucleatum* and *F. necrophorum* possess a 'classical' LPS which contains KDO, heptose and β-hydroxymyristic acid. The sugar and fatty acid compositions that we found for *F. nucleatum* LPS are essentially similar to those reported previously (Hase et al., 1977; Hofstad, 1982; Hofstad & Skaug, 1980; Kristoffersen & Hofstad, 1980).
Table 1. *Mitogenic responses of BALB/c spleen cells, thymus cells and BALB/c (nu/nu) spleen cells to purified LPSs from Fusobacterium spp.*

Spleen cells or thymocytes (5 x 10⁵ per well in 0.2 ml RPMI 1640 medium) were cultured for 48 h with or without stimulant. Values are expressed as the mean ± SD of triplicate cultures.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Dose (μg per well)</th>
<th>[3H]Thymidine uptake (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BALB/c spleen cells</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>937 ± 229</td>
</tr>
<tr>
<td>Con A</td>
<td>5</td>
<td>3945 ± 1574</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>30560 ± 6509</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19377 ± 2894</td>
</tr>
<tr>
<td><em>E. coli</em> K235</td>
<td>10</td>
<td>9595 ± 2414</td>
</tr>
<tr>
<td>LPS</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>15561 ± 2146</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td>ATCC 10953 LPS</td>
<td>10393 ± 1159</td>
</tr>
<tr>
<td></td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>3357 ± 131</td>
</tr>
<tr>
<td><em>F. necrophorum</em></td>
<td>ATCC 25286 LPS</td>
<td>10378 ± 855</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3742 ± 395</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1859 ± 225</td>
</tr>
</tbody>
</table>
LPS from Fusobacterium spp.

Fig. 2. Effects of polymyxin B on mitogenic responses of BALB/c mouse spleen cells. Cells (5 × 10⁵) were cultured for 48 h with 5 μg of Con A (○), E. coli LPS (●), F. nucleatum LPS (▲) or F. necrophorum LPS (■) in 0.2 ml RPMI 1640 medium containing various amounts of polymyxin B. Control spleen cell cultures to which no polymyxin B had been added responded as follows: Con A, 17 361 c.p.m.; E. coli LPS, 12352 c.p.m.; F. nucleatum LPS, 11486 c.p.m.; F. necrophorum LPS, 7466 c.p.m.

Fig. 3. Ability of purified LPSs from F. nucleatum and F. necrophorum to increase glucose utilization by BALB/c peritoneal macrophages. Macrophages (obtained from 6 × 10⁵ peritoneal exudate cells) were cultured with 10 μg of E. coli LPS (●), F. nucleatum LPS (▲) or F. necrophorum LPS (■) per well, or without stimulant (○), in 0.2 ml RPMI 1640 medium. Glucose consumption was assayed as described in Methods.

Table 2. Polyclonal B cell activation by purified LPSs from Fusobacterium spp.

Spleen cells (1 × 10⁶) from C3H/HeN and C3H/HeJ mice were incubated with stimulant in 0.2 ml RPMI 1640 medium supplemented with 10% (v/v) FCS. After 4 d incubation, PFC responses were determined. Values are expressed as the mean ± SD of four different cultures.

<table>
<thead>
<tr>
<th>Source of stimulant LPS</th>
<th>Dose (μg per well)</th>
<th>C3H/HeN</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>9 ± 3</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>E. coli K235</td>
<td>10</td>
<td>46 ± 5</td>
<td>6 ± 4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>69 ± 6</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>53 ± 13</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>F. nucleatum ATCC 10953</td>
<td>10</td>
<td>61 ± 7</td>
<td>6 ± 2</td>
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<tr>
<td></td>
<td>2</td>
<td>51 ± 5</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>38 ± 5</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>F. necrophorum ATCC 25286</td>
<td>10</td>
<td>53 ± 4</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>46 ± 8</td>
<td>4 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>24 ± 6</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

1970; Mashimo et al., 1985; Wollenweber et al., 1984). LPS from F. necrophorum ATCC 25286 did not contain β-hydroxyopalmitic acid, in agreement with the findings of Hofstad & Skaug (1980) for LPSs from other F. necrophorum strains.

LPSs from Fusobacterium species have been reported to possess various endotoxic activities (Garcia et al., 1975; Hawley & Falkler, 1978; Mashimo et al., 1985; Nygren et al., 1979; Svenn,
Table 3. Induction by LPSs from Fusobacterium spp. of IL-1 secretion by macrophages from C3H/HeN and C3H/HeJ mice

Macrophages (3 × 10⁶) were cultured for 24 h in the presence of stimulant in 1 ml RPMI 1640 medium. The culture supernatant (1:2 dilution; 0.1 ml) and an equal volume of C3H/HeJ thymocytes suspension (1.5 × 10⁷ ml⁻¹) were seeded into a 96-well microculture plate. Cultivation was carried out in the presence of Con A (0.2 μg per well). The basal values of [³H]thymidine incorporation were 409 ± 163 c.p.m. for C3H/HeN macrophage supernatants and 538 ± 66 c.p.m. for C3H/HeJ macrophage supernatants.
The genus *Fusobacterium* is a member of the family Bacteroidaceae (Holdeman et al., 1984). However, LPS from *Bacteroides* species has been reported to be quite different from that of *Enterobacteriaceae*, in not containing KDO, heptose or β-hydroxyxymyristic acid (Hofstad, 1982; Joiner et al., 1982; Koga et al., 1985; Nair et al., 1983; Wollenweber et al., 1980, 1984). Moreover, the biological activities of *Bacteroides* LPSs are atypical; they give weak responses in most tests of endotoxic activity (Sveen, 1977; Sveen et al., 1977), and can act as potent mitogens for spleen cells from endotoxin-nonresponder C3H/HeJ mice (Joiner et al., 1982; Koga et al., 1985; Williamson et al., 1984). Williamson et al. (1984) showed that the polysaccharide moiety of LPS from *Bacteroides* is biologically active and mediates the stimulation of B-cells from C3H/HeJ mice via macrophages. Thus, the present study reveals that *Fusobacterium* LPS differs from *Bacteroides* LPS in biological activities as well as chemical properties, although *Fusobacterium* species belong to the family Bacteroidaceae.

The 'partially purified' LPS preparations from both *Fusobacterium* strains were mitogenic for C3H/HeJ spleen cells (Fig. 1c, d). The LPS–protein complex is known to be strongly mitogenic for B cells of the C3H/HeJ mouse (Morrison et al., 1976; Sultz & Goodman, 1976). The protein content of these 'partially purified' preparations was 4–6%, suggesting that they may have been contaminated with some mitogenic proteins. In this regard, we found that an endotoxin preparation extracted from *F. nucleatum* by the butanol/water procedure reported by Morrison & Leive (1975) was rich in protein, and strongly mitogenic for C3H/HeJ spleen cells (data not shown).

The present study also revealed that LPSs from *Fusobacterium* species enhance glucose utilization by murine macrophages (Figs 3 and 4). Macrophages activated by LPS secrete various biologically active substances (Vogel & Mergenhagen, 1982). Among these substances, IL-1 possesses many activities, such as stimulation of thymocyte proliferation and activation of B cells (Dinarello, 1984). Gowen et al. (1983) showed that an IL-1-like factor stimulated bone resorption in organ cultures of mouse calvaria. In the present study, we found that the LPS preparations from *Fusobacterium* species stimulated IL-1 production by mouse peritoneal macrophages. Furthermore, we have recently found that LPS from *Fusobacterium* species stimulates bone resorption in the mouse calvaria system (unpublished data). These findings suggest that *Fusobacterium* LPS may contribute to alveolar bone loss in the development of chronic periodontitis.

Obligatory anaerobic Gram-negative filamentous rods such as *Fusobacterium* and *Bacteroides* species are opportunistic pathogens in man and animals. These types of infection are frequently of mixed aetiology. Price & McCallum (1986) demonstrated that the growth factor produced by *F. necrophorum*, which maintains the growth of *Bacteroides intermedius*, is LPS. Enhancement of the growth of oral *Bacteroides* species by *F. necrophorum* or other *Fusobacterium* species may occur in the subgingival flora in the gingival crevice of patients with periodontal diseases.

Taking these findings together, the endotoxic substances from *Fusobacterium* species may contribute to the destruction of infected tissues like the periodontium, possibly as pathogenic mediators.

**REFERENCES**


