Chemical, immunobiological and antigenic characterizations of lipopolysaccharides from *Bacteroides gingivalis* strains

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(Received 6 September 1989; revised 27 October 1989; accepted 13 November 1989)

Lipopolysaccharides (LPS) were extracted from whole cells of seven strains of *Bacteroides gingivalis* – 381, ATCC 33277, BH18/10, OMZ314, OMZ409, 6/26 and HW24D-1 – by the phenol/water procedure, and purified by treatment with nuclease and by repeated ultracentrifugation. These LPS were composed of hexoses, hexosamines, fatty acids, phosphorus and phosphorylated 2-keto-3-deoxyoctonate (KDO). The major components of the lipid portion of these LPS were hexadecanoic, 3-hydroxyhexadecanoic, branched 3-hydroxypentadecanoic and branched 3-hydroxyheptadecanoic acids. All the LPS preparations induced marked mitogenic and *in vitro* polyclonal B cell activation responses in spleen cells from both C3H/HeN and C3H/HeJ mice, exhibited no definitive preparatory activity in the local Shwartzman reaction in rabbits, but were active in the chromogenic *Limulus* amoebocyte lysate test. A monoclonal antibody (mAb) raised against the LPS from *B. gingivalis* strain 6/26 reacted with LPS from all other *B. gingivalis* strains tested. Other mAbs raised against LPS from *B. gingivalis* strains 381 and 6/26 reacted with the LPS from strains 381, ATCC 33277, BH18/10 and 6/26 (these strains were termed LPS serogroup I), as revealed by ELISA and immunodiffusion. The LPS from these strains except for 6/26 showed almost identical patterns in SDS-PAGE stained with ammoniacal silver. A mAb raised against the LPS from *B. gingivalis* HW24D-1 reacted with the LPS from strains OMZ314, HW24D-1 and OMZ409 (LPS serogroup II). These LPS, except OMZ409, exhibited very similar profiles in SDS-PAGE. These results indicate that there are at least two different antigenic groups present among LPS from *B. gingivalis* strains, as well as a common, species-specific antigen.

Introduction

Bacteroides gingivalis is a Gram-negative, anaerobic, black-pigment-forming, rod-shaped bacterium, and is suspected of being a major pathogenic organism in adult periodontitis. It has a variety of cellular and extracellular components which may modify host responses leading to the destruction of periodontal tissues (Slots & Genco, 1984; Mayrand & Holt, 1988; Slots & Listgarten, 1988). Among them, one of the most important is endotoxic lipopolysaccharides (LPS). LPS are a major component in the outer membrane of Gram-negative bacteria, and possess marked immunobiological and pharmacological activities (Daly *et al.*, 1980; Morrison, 1983; Westphal *et al.*, 1985). As compared with the 'classical' LPS from Enterobacteriaceae, LPS from *Bacteroides* species have

some different properties in terms of chemical composition and immunobiological activities. Chemically, heptose and 2-keto-3-deoxyoctonate (KDO), which are components of the core region of classical LPS, and 3-hydroxymyristic acid, a main fatty acid of endotoxic lipid A, are not detectable by the usual methods. Immunobiologically, the LPS induce activation of lymphoid cells from the classical LPS nonresponder mouse strain C3H/HeJ (Hamada *et al.*, 1988).

LPS are located on the cell surface; their polysaccharide portion is oriented toward the outside originating from within the outer membrane, and some immunodeterminant sugar residues may be easily recognized by immunocompetent cells. Thus, LPS can be highly antigenic. Schenck & Michaelsen (1987) reported that patients with periodontitis exhibited significantly higher levels of serum antibody specific for *B. gingivalis* LPS when compared to subjects with a healthy periodontium. Furthermore, periodontal treatment reduced the level of the serum antibody against *B. gingivalis* LPS (Schenck *et*

Abbreviations: Con A, concanavalin A; CI, chemical ionization; EI, electron impact ionization; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonate); LAL test, *Limulus* amoebocyte lysate test; LPS, lipopolysaccharide(s); mAb, monoclonal antibody.

al., 1987). These findings clearly suggest that *B. gingivalis* LPS play an important role not only as an immunomodulator but also as an antigen in the pathogenesis of periodontal diseases.

Because investigators have long focused mainly on two reference strains of *B. gingivalis*, strains 381 and ATCC 33277, the inter-strain differences among *B. gingivalis* LPS have not yet been characterized. We report here the determination of the chemical, immunobiological and antigenic properties of LPS from seven strains of *B. gingivalis*.

Methods

Bacterial strains and preparation of LPS. *Bacteroides gingivalis* strains 381, ATCC 33277, BH18/10, OMZ314, OMZ409, 6/26 and HW24D-1 were used in this investigation. In some experiments, *Escherichia coli* K235 grown in Trypticase soy broth (BBL) was used as a source of control LPS. *B. gingivalis* strains were cultured anaerobically in GAM broth (Nissui, Tokyo) supplemented with haemin and menadione at 37 °C. Bacterial cells were collected by centrifugation, washed twice with pyrogen-free water and lyophilized. LPS were extracted from lyophilized cells by the hot phenol/water method described by Westphal & Jann (1965). The crude extract was suspended in pyrogen-free water and collected by ultracentrifugation (100 000 g, 3 h), followed by treatment with nuclease P1 (Yamasa Shoyu Co.). Subsequently, crude LPS extracts were purified by repeated ultracentrifugation and finally lyophilized. In some experiments, LPS from oral black-pigmented and related *Bacteroides* strains such as *B. oralis* ATCC 33269, *B. loescheii* ATCC 15930, *B. intermedius* ATCC 25611 and *B. corporis* ATCC 33547 were used (Fujiwara *et al.*, 1988).

Colorimetric analysis. Total hexose was estimated by the anthrone method (Trevelyan & Harrison, 1956) with glucose as a standard. Protein was measured by using the BCA protein assay reagent (Pierce Chemical Co.) with BSA (Sigma) as a standard. Total fatty acid esters, heptose, KDO and phosphorus were quantified by colorimetric methods as previously described (Koga *et al.*, 1985). Phosphorylated KDO was quantified as follows (Kumada *et al.*, 1988). LPS from *B. gingivalis* were hydrolysed in 3 M-HCl at 100 °C for 30 min. The hydrolysates were dried *in vacuo*, suspended in 0.1 M-H₂SO₄, and assayed by the thiobarbituric acid method (Karkhanis *et al.*, 1978) with KDO (Sigma) as a standard.

GLC. For analysis of component sugars and fatty acids, LPS was hydrolysed for 6 h at 100 °C in 2 M-HCl, and the hydrolysate separated by the chloroform/methanol method (Bligh & Dyer, 1959). Neutral and amino sugars were identified as alditol acetate derivatives (Sawardeker *et al.*, 1965) by using a gas-liquid chromatograph (model GC-15A: Shimadzu) fitted with a glass column (3 mm × 2 m) of Silicone OV-225 on Chromosorb WAW DMCS (80/100 mesh; Gasukuro Kogyo, Tokyo). Fatty acids were identified as their methyl esters. The chloroform extract was treated with 4 ml 5% (w/v) HCl in methanol at 105 °C for 3 h in sealed tubes and dried under a stream of nitrogen gas. After the addition of 2% (w/v) potassium bicarbonate solution, the esters were extracted into hexane and quantified in a gas-liquid chromatograph fitted with an SPB-1 capillary column (0.25 mm × 30 m; Supelco). The component fatty acids of LPS were determined by comparison of their retention time with those of known fatty acids. Bacterial acid methyl esters CP mix (Supelco) was used as references for fatty acids.

Mass spectrometry. A model M-2000 mass spectrometer (Hitachi) and a model G-3000 gas-liquid chromatograph (Hitachi) with SPB-1 capillary column were used. Helium was the carrier gas. Electron impact ionization (EI) spectra were recorded using an ionizing energy of 70 eV and an ion-source temperature of 200 °C. Chemical ionization (CI) mass spectra were recorded with isobutane as a reactant gas and with an ion-source temperature of 140 °C.

SDS-PAGE. This was done with 4% (w/v) stacking and 12.5% (w/v) resolving gels as described previously (Fujiwara *et al.*, 1988). After electrophoresis, the gels were stained with ammoniacal silver by the method of Tsai & Frasch (1982) or with Coomassie brilliant blue.

Assay for mitogenic response and polyclonal B cell activation. C3H/HeN and C3H/HeJ mice were purchased from CLEA Japan. Spleen cells were obtained from mice aged 6–12 weeks. Mitogenic response and polyclonal B cell activation assays of C3H/HeN and C3H/HeJ mice spleen cells to LPS from *B. gingivalis* were done as described previously (Fujiwara *et al.*, 1988). Briefly, spleen cell suspensions were cultured with stimulants in RPMI 1640 medium (Gibco) with HEPES buffer (15 mM, pH 7.2) at 37 °C for 48 h in a humidified atmosphere of air/CO₂ (95:5, v/v). The cultures were pulsed for the last 6 h with 0.25 µCi (9.25 kBq) [³H]thymidine (Amersham). Incorporated [³H]thymidine was counted in an LKB scintillation counter (model 1215). Concanavalin A (Con A; Sigma) and LPS from *E. coli* K235 were used as reference mitogens.

For the polyclonal B cell activation assay, lymphoid cells were prepared from the spleens of C3H/HeN and C3H/HeJ mice by using Histopaque 1085 (Sigma). The lymphoid cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum and 2-mercaptoethanol (50 µM) for 96 h. An anti-sheep red blood cell haemolytic plaque assay was then done, as described by Cunningham & Szenberg (1968).

Limulus amoebocyte lysate (LAL) assay. A chromogenic LAL assay kit QCL-1000 (Whittaker M. A. Bioproducts) was used. LPS (1 pg dissolved in 50 µl of pyrogen-free water) was reacted with the same volume of LAL solution. The reaction mixture was incubated in flat-bottomed 96-well microtitre plates (Corning) at 37 °C for 10 min. Chromogenic substrate (100 µl) was added and reacted for 3 min. After stopping the reaction by adding 100 µl 25% (v/v) acetic acid, the absorbance at 405 nm was measured in a Titertek Multiscan photometer. The standard LPS from *E. coli* O111:B4 supplied with the assay kit was included for comparison.

Local Shwartzman reaction. This was assessed by using the dorsum of rabbits (about 3 kg body weight) as described previously (Koga *et al.*, 1987). The LPS from *B. gingivalis* and *E. coli* were suspended in pyrogen-free saline (0.85% NaCl) to a concentration of 1 mg ml⁻¹, and twofold serial dilutions were made in pyrogen-free saline and injected intradermally (200 µl) into the dorsum. After 24 h, *E. coli* K235 LPS (200 µg) was injected intravenously as a provocation dose. The diameter of the necrotic zone and the redness of the lesion were measured 24 h after the provocation.

Immunological procedures. Immunodiffusion tests were done in 1% (w/v) Noble agar (Difco). Antisera against LPS from *B. gingivalis* were obtained from rabbits as described previously (Fujiwara *et al.*, 1988) and the IgG fraction was prepared by three repeated precipitations with 33% saturation ammonium sulphate. ELISA was done as follows. LPS dissolved in 0.05 M-sodium carbonate buffer (pH 9.6) was added to wells (1 µg per well) of flat-bottomed microtitre plates (MS-3696F; Sumitomo Bakelite Co., Tokyo). Following overnight incubation at 4 °C, unbound LPS was washed off with 0.01 M-potassium phosphate buffer (pH 7.4) containing 0.8% NaCl, 0.02% KCl, 0.01% NaN₃ and 0.05% (v/v) Tween 20 (PBST). PBST was used in all washing procedures and as a diluent buffer. Then, 100 µl 1% (w/v) BSA in PBST

was added, and the plates were incubated at 25 °C for 1 h. The plate was washed twice and diluted (200–1000-fold) rabbit anti-LPS serum or undiluted hybridoma culture supernatant was added (100 µl per well). Following incubation at 37 °C for 1 h, the plates were washed a further three times and then diluted (1000-fold) alkaline-phosphatase-conjugated swine anti-rabbit immunoglobulins (Dakopatts) and alkaline-phosphatase-conjugated goat anti-mouse Ig mixture of IgG/IgA/IgM (Zymed Laboratories) were added (100 µl per well). The plates were incubated at 37 °C for 1 h, washed five times, and *p*-nitrophenyl phosphate substrate (Wako; Osaka) dissolved in diethanolamine buffer (pH 9.8; 1 mg ml⁻¹) was added (100 µg per well). The amount of colour developed after incubation at 37 °C for 1 h was measured at 405 nm by using a Titertek Multiscan photometer.

mAb production. LPS (0.1 mg) from *B. gingivalis* strains 381, 6/26 or HW24D-1 were suspended in 0.1 ml of pyrogen-free saline, mixed with an equal volume of Freund's complete adjuvant (Difco), and injected intradermally into the dorsum of BALB/c mice (CLEA Japan). This injection was repeated three times at 2-week intervals. Four days after the last immunization, spleen cells were fused to myeloma cell line Sp2/O-Ag14 with 50% (w/v) polyethylene glycol 4000. After selection of hybrid cells with HAT medium (Boehringer), the presence of anti-LPS antibodies in culture fluids was determined by ELISA as described above. The antibody-forming cultures were cloned at least twice by the limiting dilution method. The isotype and light chain of each mAb was determined by using the mouse monoclonal antibody isotyping kit (Amersham). Culture supernatants of the antibody-producing hybridomas were concentrated about 30 times by ultrafiltration using a Centriprep-30 (Amicon), and were used for reaction with LPS by immunodiffusion.

Results

Chemical composition of LPS from *B. gingivalis*

The yield of purified LPS from lyophilized whole cells of *B. gingivalis* was 0.13–0.78% (w/w). The purified preparations of LPS were composed of rhamnose, hexoses (mannose, galactose and glucose in the order of weight percentage described), hexosamines, fatty acids and phosphorus (Table 1). The total amount of hexose as

determined by the anthrone method was 21.1–66.4% (w/w). Significant amounts of KDO were not detected when LPS from *B. gingivalis* were hydrolysed in 0.1 M-H₂SO₄ at 100 °C for 30 min, but hydrolysis in 3 M-HCl released 0.6–1.1% (w/w) of phosphorylated KDO.

In addition to rhamnose and hexoses, glucosamine, galactosamine and an unknown sugar were detected by GLC. The LPS from *B. gingivalis* strains ATCC 33277, BH18/10, OMZ409, and 6/26 contained larger amounts of glucose than other *B. gingivalis* strains tested. The BCA protein assay indicated that LPS contained 2.6–6.3% (w/w) protein. However, no visible protein bands were detected by staining of SDS-PAGE slabs with Coomassie blue after electrophoresis (results not shown) of these LPS preparations (100 µg). Very low levels of heptose (<0.5%) were detected by the colorimetric method.

Analysis by GLC and GLC-MS revealed that the major fatty acids of LPS from *B. gingivalis* were hexadecanoic (C16:0), 3-hydroxyhexadecanoic (3-OH-C16:0), branched 3-hydroxypentadecanoic (branched 3-OH-C15:0) and branched 3-hydroxyheptadecanoic (branched 3-OH-C17:0) (Table 2). In contrast to straight forms of 3-hydroxy fatty acid methyl esters, the branched 3-hydroxy fatty acid methyl esters gave the same base peak (*m/e* 108) and characteristic ions such as *M*⁺ – 50 and *M*⁺ – 18 by the EI-MS. They also showed the same molecular mass by CI-MS with isobutane. However, their retention times were different from the straight forms of 3-hydroxy fatty acids in GLC. These branched fatty acids were characteristic of LPS preparations from *B. gingivalis*. Some iso-branched fatty acids such as 13-methyltetradecanoic (iso-C15:0), and 15-methylhexadecanoic (iso-C17:0) were also detected in *B. gingivalis* LPS preparations.

Table 1. Chemical composition of LPS from *B. gingivalis*

Data are expressed as the mean ± SD of at least triplicate assays. ND, Not detected.

Component*	Strain ...	Percentage (w/w) dry weight						
		381	ATCC 33277	BH18/10	6/26	OMZ314	HW24D-1	OMZ409
Total hexose ^a		25.4 ± 1.7	49.4 ± 1.8	46.1 ± 2.2	43.3 ± 1.0	21.1 ± 0.6	24.4 ± 1.6	66.4 ± 1.1
Rhamnose ^b		3.8 ± 0.3	2.0 ± 0.2	1.8 ± 0.2	1.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.2	0.5 ± 0.1
Mannose ^b		3.3 ± 0.3	2.1 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	2.6 ± 0.3	2.6 ± 0.4	0.5 ± 0.1
Galactose ^b		7.1 ± 0.5	4.9 ± 0.8	3.3 ± 0.1	2.5 ± 0.2	4.0 ± 0.3	3.2 ± 0.3	0.7 ± 0.1
Glucose ^b		5.3 ± 1.2	28.4 ± 1.3	14.4 ± 0.4	20.4 ± 3.8	3.5 ± 0.7	5.6 ± 1.0	28.7 ± 0.1
Unknown ^{b†}		0.3 ± 0.0	ND	0.7 ± 0.1	2.1 ± 0.4	1.2 ± 0.2	0.3 ± 0.1	1.7 ± 0.3
Glucosamine ^b		11.3 ± 0.9	8.3 ± 0.7	9.8 ± 0.3	6.2 ± 0.7	13.3 ± 1.4	11.3 ± 1.2	3.6 ± 0.4
Galactosamine ^b		6.2 ± 0.2	2.2 ± 0.1	2.6 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	4.6 ± 0.0	1.5 ± 0.0
Phosphorylated KDO ^a		1.0 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.6 ± 0.1
Total lipid ^a		23.3 ± 0.2	13.4 ± 0.1	8.7 ± 1.9	17.6 ± 1.4	14.0 ± 1.1	22.6 ± 2.9	6.8 ± 1.1
Phosphorus ^a		2.8 ± 0.0	1.7 ± 0.0	1.9 ± 0.0	2.3 ± 0.1	3.0 ± 0.0	3.2 ± 0.2	0.5 ± 0.0
Protein ^a		3.1 ± 1.1	6.3 ± 1.1	3.4 ± 0.0	3.3 ± 0.7	3.1 ± 0.0	2.6 ± 0.2	6.3 ± 0.5

* a, Quantified by colorimetric analysis; b, quantified by GLC.

† Calculated by assuming that the response adjustment factor of the unknown sugar was the same as that of glucose with GLC.

Table 2. Fatty acid composition of LPS from *B. gingivalis*

Fatty acid composition was determined by GLC and GLC-MS. Data are expressed as the mean $\pm$ SD of at least triplicate assays. ND, Not detected; tr., trace amount ($<1\%$).

Fatty acid	Strain ...	Percentage total fatty acids						
		381	ATCC 33277	BH18/10	6/26	OMZ314	HW24D-1	OMZ409
iso-C15:0		2.3 $\pm$ 0.0	1.0 $\pm$ 0.2	tr.	tr.	1.4 $\pm$ 0.5	1.1 $\pm$ 0.2	ND
C16:0		18.2 $\pm$ 2.1	14.4 $\pm$ 0.1	16.3 $\pm$ 0.5	19.3 $\pm$ 0.7	14.1 $\pm$ 1.3	17.9 $\pm$ 0.1	17.9 $\pm$ 1.4
iso-C17:0		3.3 $\pm$ 0.6	5.7 $\pm$ 1.8	4.9 $\pm$ 1.4	2.6 $\pm$ 0.4	6.1 $\pm$ 1.8	3.0 $\pm$ 0.2	3.3 $\pm$ 1.0
C18:1		1.7 $\pm$ 0.5	1.2 $\pm$ 0.5	tr.	1.2 $\pm$ 0.5	ND	tr.	2.6 $\pm$ 1.2
3-OH-C16:0		9.5 $\pm$ 2.1	7.9 $\pm$ 1.6	7.7 $\pm$ 1.7	19.1 $\pm$ 2.5	8.1 $\pm$ 1.8	16.0 $\pm$ 2.6	6.7 $\pm$ 1.9
branched 3-OH-C15:0		10.8 $\pm$ 0.9	12.0 $\pm$ 1.0	12.7 $\pm$ 1.1	9.3 $\pm$ 1.7	15.6 $\pm$ 0.6	10.3 $\pm$ 1.5	20.8 $\pm$ 1.4
branched 3-OH-C16:0		3.1 $\pm$ 0.5	3.8 $\pm$ 0.9	4.3 $\pm$ 0.9	3.6 $\pm$ 0.5	4.0 $\pm$ 0.8	3.9 $\pm$ 0.8	3.4 $\pm$ 0.7
branched 3-OH-C17:0		46.0 $\pm$ 3.6	50.6 $\pm$ 4.2	49.0 $\pm$ 4.2	42.6 $\pm$ 7.5	48.4 $\pm$ 1.9	41.5 $\pm$ 6.1	36.6 $\pm$ 2.5

SDS-PAGE of LPS from *B. gingivalis*

Silver-stained profiles of the LPS preparations from the seven strains of *B. gingivalis* are shown in Fig. 1. Each showed a series of closely spaced bands in a ladder pattern. The LPS profiles of strains 381, ATCC 33277 and BH18/10, and of strains OMZ314 and HW24D-1 were almost identical, within each cluster of strains. Although the profiles of 6/26 and OMZ409 were different from the two clusters mentioned above, they showed some similarity to those of strain 381 and strain OMZ314, respectively.

Immunobiological activities of LPS from *B. gingivalis*

LPS preparations from all the *B. gingivalis* strains induced strong mitogenic responses and *in vitro* polyclonal B cell activation responses in cultures of spleen cells from both LPS-nonresponder C3H/HeJ mice (Fig. 2) and LPS-responder C3H/HeN mice (results not shown). The response patterns of spleen cells from these mice were essentially identical. In this connection, it was noted that *E. coli* K235 LPS induced marked responses in C3H/HeN spleen cell cultures but not in C3H/HeJ cell cultures.

In the LAL test, *B. gingivalis* strains possessed activities comparable to LPS of *E. coli* (Table 3). In the Shwartzman test, preparatory and provocative injection of *E. coli* K235 LPS induced a necrotic lesion at doses of more than 12.5 μ g, and inflammatory lesions were observed at lower doses of LPS ($<0.785 \mu$ g). However, LPS from *B. gingivalis* exhibited no Shwartzman preparatory activity (Table 3).

Antigenic properties of LPS from *B. gingivalis*

A total of 10 hybridomas producing mAbs to LPS from *B. gingivalis* 381, 6/26 and HW24D-1 were obtained; the

Table 3. Chromogenic LAL test and local Shwartzman activity of LPS from *B. gingivalis*

	LAL test (A_{405})	Shwartzman reaction (minimum dose, μ g)*
<i>E. coli</i>	1.530†	12.5‡
<i>B. gingivalis</i>		
381	0.703	> 200
ATCC 33277	0.779	> 200
BH18/10	0.720	> 200
6/26	0.913	> 200
OMZ314	0.743	> 200
HW24D-1	0.601	> 200
OMZ409	0.685	> 200

* The minimum preparatory dose was determined as described in Methods; doses from 0.785–200 μ g were tested.

† LPS from *E. coli* O111:B4.

‡ LPS from *E. coli* K235.

cell lines were named 381-1, 381-2, 381-3, 6/26-1, 6/26-2, 6/26-3, 6/26-4, 6/26-5, 6/26-6 and 24D1, respectively. The isotypes of mAbs 381-1, 381-2 and 6/26-6, mAbs 6/26-1, 6/26-2, 6/26-3, 6/26-4 and 6/26-5, and mAb 24D1 were identified as IgG1, IgG3 and IgM, respectively. All mAbs were found to produce a κ light chain. ELISA revealed that mAb 381-1 reacted with only the LPS from strain 381, while mAb 381-2 reacted with that from 381 and ATCC 33277 (results not shown). The reaction patterns of mAbs 381-3, 6/26-2, 6/26-4 and 24D1 are shown in Fig. 3. The LPS from *B. gingivalis* strains 381, ATCC 33277, BH18/10 and 6/26 reacted with both mAbs 381-3 and 6/26-2. mAb 6/26-4 reacted with LPS from all the strains of *B. gingivalis* tested. The mAb 6/26 LPS series, except mAb 6/26-4, showed similar reaction patterns to 6/26-2 (results not shown). However, mAb 24D1 reacted only with the LPS from OMZ314, HW24D-1 and OMZ409. The response of OMZ314 LPS was strongest and that of OMZ409 was weakest. No

Fig. 1. Silver-stained gel after SDS-PAGE of LPS from seven strains of *B. gingivalis*. Electrophoresis was done in a 12.5% (w/v) acrylamide gel, containing LPS from *B. gingivalis* strains 381 (lane 1), ATCC 33277 (lane 2), BH18/10 (lane 3), 6/26 (lane 4), OMZ314 (lane 5), HW24D-1 (lane 6) and OMZ409 (lane 7).

mAbs were found to react with LPS from strains of *B. oralis*, *B. loescheii*, *B. intermedius* and *B. corporis*.

Immunodiffusion patterns of the LPS preparations from *B. gingivalis* with the concentrated mAbs 381-3, 6/26-2 and 6/26-4 are shown in Fig. 4. The LPS from *B. gingivalis* strains 381, ATCC 33277, BH18/10 and 6/26 formed a fused precipitin band with mAbs 381-3 and 6/26-2. mAb 6/26-4 formed a fused precipitin band with LPS from all the *B. gingivalis* strains. No precipitin band was observed with mAbs 381-1, 381-2, 6/26-6 and 24D1. mAbs 6/26-1, 6/26-3 and 6/26-5 showed very similar immunodiffusion patterns to mAb 6/26-2 (results not shown).

Discussion

In previous studies it has been demonstrated that chemical and immunobiological properties of LPS from *Bacteroides* species were different from those of other oral periodontal pathogens such as *Actinobacillus actinomycescomitans* and species of Enterobacteriaceae (Koga *et al.*, 1985, 1987; Hamada *et al.*, 1988; Fujiwara *et al.*, 1988). LPS from *B. gingivalis* has some chemical features in common with LPS of other oral and non-oral *Bacteroides* species (Nair *et al.*, 1983; Weintraub *et al.*, 1985).

The absence of KDO in LPS from *Bacteroides* species has been reported by several groups (Hofstad, 1974; Kasper *et al.*, 1983; John & Bryn, 1985). Our analyses of LPS from *B. gingivalis* (Koga *et al.*, 1985) and other black-pigmented and related *Bacteroides* (unpublished

Fig. 2. Mitogenic responses (a) and polyclonal B cell activation responses (b) of spleen cells from C3H/HeJ mice to LPS from *B. gingivalis*. For determination of mitogenic responses, spleen cells (7×10^5 per well) from C3H/HeN and C3H/HeJ mice were incubated with 10 μ g ($\square$), 5 μ g ($\blacksquare$) and 1 μ g ($\boxtimes$) LPS or Con A per well or without LPS ($\square$) in RPMI 1640 medium supplemented with 15 mM-HEPES buffer (pH 7.2) at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air. Cultures were pulsed with 9.25 kBq [³H]thymidine during the last 6 h of a 48 h incubation period. For determination of polyclonal B cell activation responses, lymphoid cells were isolated from spleen cells of C3H/HeN and C3H/HeJ mice. The cells (1×10^6 per well) were incubated with 10 μ g ($\square$), 1 μ g ($\blacksquare$) and 0.1 μ g ($\boxtimes$) LPS per well or without LPS ($\square$) in RPMI 1640 medium supplemented with 2-mercaptoethanol (50 μ M) and 10% (v/v) foetal calf serum at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air. After 4 d incubation, anti-sheep erythrocyte plaque-forming cell responses were determined. Values are expressed as the mean $\pm$ SD of three different cultures.

data) revealed only trace amounts of KDO by the thiobarbituric acid method of Karkhanis *et al.* (1978), and the colour developed was atypical. Therefore, we considered that the LPS of these *Bacteroides* contained no significant amounts of KDO. However, the study by Kumada *et al.* (1988) indicated that KDO became detectable by the thiobarbituric acid assay if LPS was pretreated with 3 M-HCl at 100 °C for 30 min. The authors concluded that *Bacteroides* LPS possess KDO that is substituted with phosphate in the 5-O- position of

Fig. 3. ELISA of LPS from *B. gingivalis* strains 381, ATCC 33277, BH18/10, 6/26, OMZ314, HW24D-1 and OMZ409 with four mAbs raised against the LPS from *B. gingivalis* strains 381, 6/26 and HW24D-1. (a) mAb 381-3; (b) mAb 6/26-2; (c) mAb 6/26-4; (d) mAb 24D1. Undiluted culture supernatant of each hybridoma clone was used as mAb.

Fig. 4. Immunodiffusion pattern of LPS from *B. gingivalis* with mAbs against LPS from *B. gingivalis* strains 381 (mAb 381-3, centre well A), and 6/26 (mAb 6/26-2, centre well B; mAb 6/26-4, centre well C). The outer wells contained 10 μ l *B. gingivalis* LPS solutions (1 mg ml^{-1}) from strains 381 (1), ATCC 33277 (2), BH18/10 (3), 6/26 (4), OMZ314 (5), HW24D-1 (6) and OMZ409 (7).

KDO. In this study, we followed their method and detected significant amounts of KDO. In this regard, Beckmann *et al.* (1989) noted that mild acid hydrolysis of the dephosphorylated LPS of *B. fragilis* that had been treated with aqueous 50% hydrofluoric acid released KDO.

The fatty acid analyses revealed that 3-hydroxytetradecanoic acid (3-OH-C14:0), a major component of enterobacterial LPS, was not detected in LPS from any of the *B. gingivalis* strains (Table 2), nor in other *Bacteroides* species (Weintraub *et al.*, 1985). The major fatty acids of LPS from *B. gingivalis* were branched 3-hydroxypenta-

decanoic (branched 3-OH-C15:0) and branched 3-hydroxyheptadecanoic (branched 3-OH-C17:0). The base peak and fragment ion peaks of their methyl esters in the mass spectrum were characteristic of 3-hydroxy fatty acid methyl esters (Ryhage & Stenhagen, 1960). However, the branching site of these fatty acids is not yet known. As no fragment ion peaks specific for the branch were detected, it is suggested that the branching site is separate from the carboxyl radical. Wollenweber *et al.* (1980) detected 3-hydroxy-15-methyl-hexadecanoic acid (3-OH-iso-C17:0) in the LPS from *B. fragilis* and four other *Bacteroides* species, a report confirmed by other investigators (Kasper *et al.*, 1983; Nair *et al.*, 1983; Mashimo *et al.*, 1985; Johne & Bryn, 1986). We have found these branched 3-hydroxy fatty acids in the LPS from oral *Bacteroides* species such as *B. oralis* ATCC 33269, *B. loescheii* ATCC 15930, *B. intermedius* ATCC 25611 and *B. corporis* ATCC 33457 (unpublished data). Thus, it appears that the presence of such fatty acids is a special feature of the LPS from *Bacteroides* species.

The LPS from all strains of *B. gingivalis* possessed mitogenic and polyclonal B cell activation activities in spleen cell cultures from C3H/HeN mice as well as LPS nonresponder C3H/HeJ mice. This finding is an extension of the previous report indicating that the lipid portion isolated from *B. gingivalis* ATCC 33277 LPS by hydrolysis in 1 M-HCl at 100 °C for 1 h was composed of fatty acids (70.5%, w/w) and glucosamine (22.5%, w/w), and exhibited mitogenic activity against both C3H/HeN and C3H/HeJ mice (Fujiwara *et al.*, 1988). Toxicities determined by the local Shwartzman reaction were very weak, supporting previous work (Sveen, 1977; Mansheim *et al.*, 1978; Johne & Bryn, 1986; Koga *et al.*, 1987). However, activities in the chromogenic LAL assay were of the same order as LPS from *E. coli*. It has been reported that the biologically active entity of enterobacterial LPS is lipid A (Westphal *et al.*, 1985). The fatty acid composition of LPS of *B. gingivalis*, with its branched 3-hydroxy fatty acids, differs from lipid A of *E. coli*, and may contribute to the difference in biological activities. Different chemical structures of the core region of LPS may also modulate the biological activities.

mAb 6/26-4 reacted with the LPS from all seven strains of *B. gingivalis*. Furthermore, immunodiffusion using rabbit polyclonal antisera raised against LPS from the seven test strains displayed a fused precipitin band with all *B. gingivalis* LPS examined. Neither mAbs nor rabbit antiserum raised against LPS from *B. gingivalis* reacted with LPS from other oral *Bacteroides* species. These results clearly indicated that there was a species-specific antigen in the LPS from *B. gingivalis* strains. Millar *et al.* (1987) reported that one mAb raised against

B. gingivalis 381 whole cells gave specific reactions against LPS from strain 381 and three other strains of *B. gingivalis*, but not against LPS from other *Bacteroides* species and species of Enterobacteriaceae. Using rabbit antisera to *B. gingivalis* whole cell serum, Okuda *et al.* (1986) found that LPS was one of the species-specific antigens among black-pigmented oral *Bacteroides* species. Using rabbit antiserum to *Bacteroides* LPS, we obtained similar results (Fujiwara *et al.*, 1988).

The LPS from *B. gingivalis* strains 381, ATCC 33277 and BH18/10 formed a fused precipitin band with each other against rabbit anti-LPS serum and mAb 381-3. These results indicated that the antigenicity of the LPS from the three strains was very similar. Furthermore, the LPS from *B. gingivalis* 6/26 reacted with mAb 381-3, and the mAb 6/26 series, except 6/26-4, reacted with the LPS not only from strain 6/26, but also with LPS from the three strains mentioned above. Conversely, mAb 24D1 reacted with the LPS from *B. gingivalis* strains OMZ314, HW24D-1 and OMZ409, but not with the LPS from *B. gingivalis* strains 381 and 6/26. These results suggest that the *B. gingivalis* strains tested form at least two LPS serogroups, depending on their antigenic specificities as revealed by mAbs to *B. gingivalis* LPS. LPS serogroup I includes strains 381, ATCC 33277, BH18/10 and 6/26, while LPS serogroup II contains strains OMZ314, HW24D-1 and OMZ409. It should be noted here that the metabolic properties of *B. gingivalis* strains in terms of sugar fermentation and the production of phenylacetic acid were almost identical. In addition, the chemical and biological properties of the LPS from serogroups I and II were essentially similar.

Recently, we have found intra-species differences in the serological specificity of the fimbrial protein in *B. gingivalis* (unpublished). Some mAbs raised against chromatographically purified fimbriae from *B. gingivalis* 381 were found to react with the fimbriae from *B. gingivalis* strains 381, ATCC 33277 and BH18/10, but not with those from strains OMZ314, HW24D-1, OMZ409 and 6/26. It is interesting that the serological heterogeneity of *B. gingivalis* LPS generally correlates with that of the fimbriae among the *B. gingivalis* strains, except for strain 6/26. Although strain 6/26 belongs to LPS serogroup I, the immunological specificity of the fimbriae of strain 6/26 is different from that of strain 381. Also, the SDS-PAGE profile of LPS from *B. gingivalis* strains 381, ATCC 33277 and BH18/10 is almost identical, but that of 6/26 LPS is somewhat different. Furthermore, an absorption experiment using rabbit polyclonal antibodies indicated that anti-381 LPS serum absorbed with strain 381 LPS reacted with neither 381 nor 6/26 LPS. Conversely, when anti-6/26 LPS serum was absorbed with 6/26 LPS, its reactivity to strain 6/26 LPS disappeared, while its reactivity to strain 381 LPS

remained (data not shown). These results clearly suggest that strain 6/26 LPS has other antigenic determinant(s) which are lacking in strain 381 LPS.

This study was supported in part by grants-in-aid 01480430 and 01044085 for scientific research from the Ministry of Education, Science and Culture of Japan.

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