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Modulation of Bone Metabolism by Two Chemically Distinct Lipopolysaccharide Fractions from *Bacteroides gingivalis*

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Two separate species of lipopolysaccharide (LPS) from *Bacteroides gingivalis* 381 have been isolated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated not only the heterogeneity of each species, but also that they represented high- and low-molecular-weight LPS entities. Although both contained the same carbohydrate and fatty acid components, the proportions of these differed between the LPS species. The direct effects of these two species in modulation of bone resorption and bone collagen and noncollagen protein synthesis have been examined. In a bone resorption assay, these two species stimulated ^{45}Ca release from prelabeled fetal rat bones in a concentration range of 0.5 to 3.0 μg/ml. The two LPS species also elicited a 30 to 40% reduction in collagen protein formation at 10 μg/ml. Responses of the same order of magnitude were observed with LPS from *Salmonella minnesota* at 10 μg/ml. The higher-molecular-weight LPS species also significantly inhibited noncollagen protein formation. This is the first report that LPS from *B. gingivalis* 381, a suspected periodontal pathogen, inhibits bone collagen formation and, in conjunction with the bone resorption potency, further implicates LPS in alveolar bone loss associated with periodontal disease.

The correlation between the incidence of *Bacteroides gingivalis* and alveolar bone loss is well documented (19, 22, 24). High concentrations of this organism in the subgingival microflora strongly implicate it as a periodontal pathogen (20). Numerous bacterial surface components and end products of growth and metabolism from *B. gingivalis* have been implicated as virulence factors which may be involved in host tissue destruction in periodontal disease (21). One of these, lipopolysaccharide (LPS), has been shown to stimulate bone resorption in organ culture (5, 13). Although the biological potency of LPS from *B. gingivalis* is low in comparison to LPS from aerobic gram-negative organisms when assessed by lymphocyte mitogenicity, pyrogenicity (13), chicken embryo lethality, and the Schwartzmann test (8), its potency in stimulating bone resorption in organ culture is high.

This study explores the mechanisms whereby two chemically distinct LPS fractions from *B. gingivalis* modulate bone metabolism in organ culture. In this regard, we report for the first time the effect of *B. gingivalis* LPS fractions on one parameter of bone metabolism, namely, bone formation.

MATERIALS AND METHODS

Purification of LPS fractions. *B. gingivalis* strain 381 (an oral isolate provided by S. Socransky, Forsyth Dental Center, Boston, Mass.) was grown to early stationary phase in tryptic soy broth supplemented with hemin (5 mg/liter) and menadione (1 mg/liter). The purity of cultures was verified by phase-contrast microscopy, Gram staining, and subculturing on blood agar plates. Bacteria were harvested by centrifugation (12,000 × *g* for 20 min), washed three times with 0.15 M NaCl, and lyophilized. LPS was prepared by modifying the methods previously described (13). Lyophilized cells were suspended (10 to 15%, wt/vol) in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM disodium EDTA, pasteurized by incubation at 60°C for 30 min, and then broken in a Braun homogenizer (five 1-min bursts) at 4°C. The glass beads were removed by centrifugation at 200 × *g*. The cell suspension was centrifuged at 12,000 × *g* for 20 min, and the resulting supernatant was ultracentrifuged at 80,000 × *g* for 2 h. The pellet was resuspended in distilled water and centrifuged at 12,000 × *g* for 20 min, and the supernatant was centrifuged at 80,000 × *g* for 2 h. This pelleted membrane fraction was lyophilized and next extracted with phenol at 68°C (27). Briefly, the membrane fraction was suspended in distilled water (10 mg/ml) and heated to 65 to 68°C, an equal volume of 90% phenol (preheated to 65 to 68°C) was added, and the mixture was stirred vigorously for 15 min at 65 to 68°C. This mixture was cooled on ice to 10°C and then centrifuged at 10,000 × *g* for 30 min to separate the two phases. The aqueous phase was removed, and the phenol phase was re-extracted at 65 to 68°C with an equal volume of water for 15 min as above. The two phases were cooled and separated by centrifugation. The aqueous phases were combined, dialyzed against distilled water at 4°C to remove the dissolved phenol, and then lyophilized. The lyophilized material was resuspended in distilled water and centrifuged at 100,000 × *g* for 9 h at 4°C, and the pellet was lyophilized. Portions of this pellet were resuspended (25 to 30 mg/5 ml) in 0.1 M Trishydrochloride buffer, pH 8.0, containing 1 mM disodium EDTA and 0.3% sodium deoxycholate and then chromatographed on columns (1.7 by 90 cm) of Sephadex G-100 equilibrated in the same buffer. Fractions of 3 ml were collected at room temperature at a flow rate of 10 ml/h and analyzed for neutral sugars by the anthrone reaction (18). The fractions corresponding to the different carbohydrate-containing peaks (see Fig. 1) were pooled and dialyzed first against distilled water and then water containing Dowex 1-X8 (10 g/liter). Lyophilized peaks were resuspended in distilled water, electrodialyzed (2), neutralized with sodium hydroxide, and lyophilized.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10%

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TABLE 1. Recovery of LPS from *B. gingivalis* 381

Fraction	Dry wt (mg)	% Cell	% Membrane	% Carbohydrate	% Fatty acids	% Protein
Lyophilized cells	12.2 g	100.0				
Membrane prepn	777.0	6.4	100.0			
Aqueous phase from phenol-water extraction	187.6	1.5	24.1			
Ultracentrifuge pellet	131.9	1.1	17.0			
Sephadex G-100						
Peak 1	63.1	0.53	8.1	75.1	0.5	1.8
Peak 2	28.9	0.24	3.7	33.2	3.8	2.5
Peak 3	33.6	0.28	4.3	18.1	13.9	1.5

slab gels (14 by 14 cm) (7). Components were detected by staining with silver nitrate (11).

Chemical analyses. For neutral and amino sugars, samples were hydrolyzed with 2 N HCl at 100°C for 6 h, separated on coupled columns of Dowex 50 and Dowex 1, converted to their alditol acetate derivatives (17), and then quantitated on a Varian model 3700 gas chromatograph interfaced to an Apple IIe computer through a Gilson model 620 data module. Neutral and amino sugars were resolved on columns (2 mm by 2 m) of OV-225 and OV-17 (Supelco, Bellefonte, Pa.), respectively. For fatty acid analyses, samples were hydrolyzed with 4 N HCl at 100°C for 5 h and then extracted with petroleum ether (30 to 60°C). Released fatty acids were methylated by boron trichloride-methanol treatment (23) and then quantitated on the computer-interfaced Varian model 3700 gas chromatograph. Fatty acids were separated on columns (2 mm by 2 m) of 3% SP 2100-DOH (Supelco) under temperature program conditions (144 to 225°C at 4°C/min), with nitrogen as the carrier gas (20 ml/min). Analyses of fatty acid samples by mass spectroscopy were conducted on a Hewlett-Packard 5992 gas chromatograph-mass spectrometer system under the same conditions as above. For amino acid analysis, samples were hydrolyzed with 6 N HCl at 105°C for 28 h and then quantitated as their *o*-phthaldehyde derivatives (6) by a high-pressure liquid chromatograph interfaced to an Apple IIe computer through a Gilson model 620 data module. *o*-Phthaldehyde-amino acids were separated on Ultrasphere ODS columns (4.6 by 250 mm) at 37°C, using a 0.1 M sodium acetate (pH 7.2)–methanol elution gradient (0 to 100%), and detected with a Gilson model 121 fluorescence detector (excitation, 305 to 395 nm; emission filter, 450 nm).

Bone resorption assay. Bone resorption was quantified by the release of ^{45}Ca from prelabeled fetal rat radii and ulnae by the technique previously described in detail by Raisz and Niemann (16) and Nair et al. (13). The amount of ^{45}Ca in the medium and in the bones was determined, and bone resorption was expressed as a percentage of the total ^{45}Ca released into the medium.

Bone formation assay. Bone collagen protein (CP) and noncollagen protein (NCP) formation was determined by a modification of a technique described by Canalis and coworkers (1). The flat portions of the frontal and parietal bones form 21-day fetal rat calvaria were removed and split along the sagittal suture. Individual half calvaria were then placed in sterile 25-ml flasks containing BGJ medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with bovine serum albumin, nonessential amino acids, and 1 mM unlabeled proline. Various concentrations of LPS fractions or insulin (positive control) were introduced at this time. These were cultivated for 24 h in an atmosphere of 5% CO_2–air at 37°C. At 22 h, the bones were pulsed with 5 μCi of labeled proline ([^{14}C]proline, 250 mCi/mmol; New England Nuclear, Boston, Mass.). To minimize variation, one-half of the calvaria was used for incubation with the experimental agent and the other half served as the control. After incubation, the calvaria were washed, dried, and weighed. The calvaria were then homogenized in 0.5 M acetic acid, dialyzed with Spectrapor 3 tubing, and lyophilized. The lyophilized material was hydrolyzed in 6 N HCl at 105°C for 28 h, and the labeled proline and hydroxyproline were separated by descending paper chromatography in the solvent system isopropanol-acetic acid-water (75:10:15, vol/vol/vol) for 24 h at room temperature on Whatman 3MM chromatography paper. The chromatograms were cut into strips and radioactivity was measured on a Beckman LS 7000 liquid scintillation spectrometer. Values for [^{14}C]hydroxyproline and [^{14}C]proline were always greater than 10 times the background. The formation of [^{14}C]hydroxyproline (dpm per microgram [dry weight] of bone) was used as a measure of CP synthesis. Assuming bone collagen has a hydroxyproline/proline ratio of 1 (4), NCP formation was calculated as follows: NCP = (dpm of [^{14}C]proline − dpm of [^{14}C]hydroxyproline)/(microgram [dry weight] of bone). This equation also assumes equivalent specific activities for [^{14}C]hydroxyproline and [^{14}C]proline in bone collagen.

RESULTS

To improve LPS recovery and enhance cell breakage, Braun homogenization was utilized for cell disruption instead of repeated passages through a 25-gauge needle. This modified membrane preparation constituted 6.4% of the cell (Table 1). After phenol extraction, the aqueous phase could be resolved into three distinct peaks by gel filtration on Sephadex G-100 in the presence of sodium deoxycholate (Fig. 1). Of the total material applied to the column, 50.2% was recovered as peak 1, 23% as peak 2, and 26.8% as peak 3. The carbohydrate and fatty acid contents of these three peaks are shown in Table 2. Peak 1 was predominantly carbohydrate. The small amount of fatty acids associated with peak 1 were primarily $C_{16:0}$ and $C_{18:0}$ constituents. In contrast, the carbohydrate and fatty acid components of peaks 2 and 3, while characteristic of *Bacteroides* sp. LPS (13), were chemically quite different from each other. Peak 2 contained more galactose and galactosamine than peak 3 but less mannose and glucosamine. The fatty acids were identified as even, odd, and branch chained, with evidence from mass spectroscopy of two fatty acids hydroxylated in the 3 position. Peak 3 contained significantly more iso-branched $C_{15:0}$ fatty acids than peak 2. There was no 3-hydroxytetradecanoate detected in the LPS fractions. Protein con-

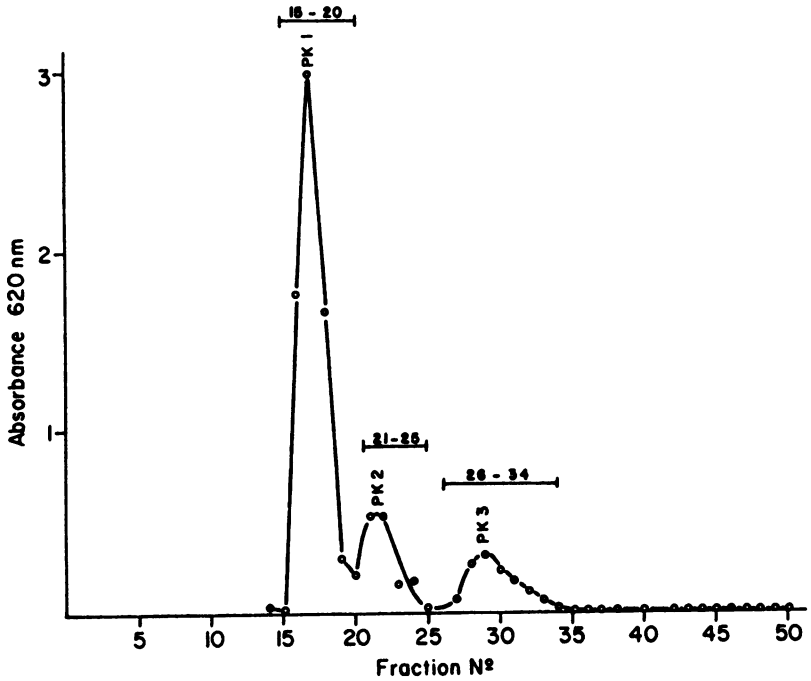

FIG. 1. Gel filtration on Sephadex G-100 of the aqueous phase from phenol-extracted membranes of *B. gingivalis*. Conditions are described in Materials and Methods. Fractions were analyzed for neutral sugars by the anthrone reaction.

tent, assessed by amino acid analysis, was 2.5 and 1.5% for peaks 2 and 3, respectively.

SDS-PAGE on 10% gels stained with silver nitrate showed peaks 2 and 3 to be two separate species, each of which was resolved into several components. The multiple banding pattern seen for peak 3 was characteristic for LPS (14) (Fig. 2). The peak 2 component banding pattern is not well resolved and is probably a reflection of both the nature of the LPS itself and the sensitivity of the silver stain. A bovine serum albumin standard, run at the level of protein contamination present in peak 2 LPS, was not detected by silver stain in the development time required for the LPS preparation (data not shown). Consequently, the bands detected on the gels are not due to the protein contamination of the LPS preparation.

When examined in a bone resorption assay, the LPS fractions from *B. gingivalis* stimulated ^{45}Ca release from prelabeled fetal rat bones in the concentration range of 0.5 to

TABLE 2. Chemical composition of *B. gingivalis* fractions

Component	% Composition		
	Peak 1	Peak 2	Peak 3
Sugar[a]			
Rhamnose	6.0	17.4	13.8
Mannose		8.9	29.3
Galactose		24.6	9.4
Glucose	93.8	24.6	21.5
Glucosamine	0.1	14.6	26.0
Galactosamine	0.1	9.9	
Fatty acid[b]			
$C_{14:0}$	3.9	2.1	2.6
$C_{a15:0}$		12.0	33.0
$C_{15:0}$	1.4		
$C_{16:0}$	63.7	29.0	27.8
C_{X-1}[c]		12.2	8.5
C_{X-2}[d]		14.0	7.6
C_{X-3}[d]		30.5	20.7
$C_{18:0}$	31.0		

[a] Percentage of total carbohydrate.

[b] Percentage of total fatty acids; all values were calculated with a molar response factor of 1.

[c] Exhibited an *m/e* of 74 and 87 on gas chromatography-mass spectroscopy, characteristic of a nonhydroxylated fatty acid (9). Based on retention time, X-1 was tentatively identified as a C17 fatty acid.

[d] Exhibited an *m/e* of 74 and 103, characteristic of a 3-hydroxyl fatty acid (9). Based on retention time, X-2 and X-3 were tentatively identified as C_{16} and C_{17} fatty acids, respectively.

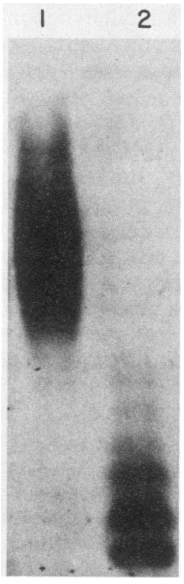

FIG. 2. SDS-PAGE on 10% gels of the LPS fractions eluted from Sephadex G-100 (Fig. 1). Lane 1, Peak 2 (5 μg); lane 2, peak 3 (10 μg). Gels were stained with silver nitrate.

TABLE 3. Bone resorption activity of *B. gingivalis* fractions[a]

Treatment	Concn (μg/ml)	% Total ^{45}Ca released
Control		18.3 ± 0.4
S. minnesota LPS[b]	3	62.9 ± 5.4[c]
	1	50.4 ± 4.9[c]
	0.5	37.9 ± 4.1[c]
B. gingivalis peak 1	3	19.8 ± 0.7
B. gingivalis peak 2	3	83.9 ± 2.4[c]
	1	66.1 ± 9.4[c]
	0.5	41.0 ± 4.5[c]
B. gingivalis peak 3	3	75.5 ± 5.5[c]
	1	41.1 ± 1.1[c]
	0.5	26.0 ± 2.4[c]

[a] Values are means ± standard errors for eight bones cultured for 5 days.
[b] LPS purified from *S. minnesota* was a generous gift from Otto Luderitz (Max-Planck-Institut für Immunobiologie, Freiberg, Federal Republic of Germany).
[c] Significantly different from control, $P < 0.01$.

3.0 μg/ml (Table 3). A similar dose-response relationship was seen with LPS from *Salmonella minnesota*. On the other hand, polysaccharide-enriched peak 1 did not stimulate ^{45}Ca release.

The effect of the *B. gingivalis* LPS fractions in a bone formation assay was also explored by determining the incorporation of [^{14}C]proline into CP and NCP of fetal rat calvaria. Insulin (10^{-8} M) stimulated the formation of bone collagen (CP) as previously reported (1) and served as the positive control (Table 4). Peaks 2 and 3 at 10 μg/ml elicited a 30 to 40% reduction in CP formation compared with controls. Peak 2 also significantly inhibited NCP formation. A comparable inhibition of CP formation was also obtained with LPS (10 ug/ml) from *S. minnesota*.

DISCUSSION

LPS from *B. gingivalis* has been separated into two species, designated peaks 2 and 3, by elution from columns of Sephadex G-100 under disagreggation conditions. These fractions constituted 0.24 and 0.28% of the dry weight of the cell, respectively. Although peaks 2 and 3 contained similar carbohydrate and fatty constituents, the relative proportions of these residues in the two LPS fractions were significantly different. The lower ratio of carbohydrate/fatty acids in peak 3 suggests that this lower-molecular-weight species has smaller carbohydrate side chains associated with the lipid moiety. In contrast, the higher-molecular-weight species in peak 2 with its higher carbohydrate/fatty acid ratio likely contains more complete carbohydrate side chains. Such heterogeneity is also consistent with the banding patterns seen on SDS-PAGE with these LPS fractions. Peak 1, eluting at the void volume on the Sephadex G-100 columns, constituted 0.53% of the cell. Analysis of peak 1 indicated that it was predominantly glucose and rhamnose, with only trace amounts of hexosamines and fatty acids. Peak 1 did not exhibit any activity in the bone resorption assay. Thus, on the basis of the chemical and biological data, this component is not an LPS.

The high potency of the *B. gingivalis* LPS fractions in stimulating bone resorption confirms findings previously reported (5, 13). While previous studies have demonstrated that cell wall fractions from gram-positive organisms such as *Streptococcus mutants* and *Streptococcus sanguis* (10) and muramyl dipeptide (25) have been shown to inhibit bone protein synthesis in calvarial systems (10), this is the first report to indicate that LPS can inhibit bone collagen formation. Interestingly, the higher-molecular-weight species of *B. gingivalis* LPS also inhibited NCP formation in bone. While it is likely that this inhibition is a reflection of a decrease in protein synthesis, other explanations are possible. For example, LPS may decrease uptake of labeled amino acids by calvarial cells or increase the size of the free pool of proline within the cells. Alternatively, the degradation of newly synthesized protein may be enhanced by LPS. However, the finding that LPS inhibits the labeled proline incorporation into CP more consistently than into NCP makes these latter two possibilities less likely.

The mechanism(s) for the modulation of bone metabolism by LPS is not well characterized. However, current evidence implicates both prostaglandins (3, 12) and interleukine 1 (26) as potential mediators of LPS effects on bone metabolism. Both have been shown to have a bimodal effect on collagen synthesis in bone (15; E. Canalis, Am. Soc. Bone Miner. Res., in press). Therefore, it is probable that LPS, when examined over a wider range of concentrations, will demonstrate a bimodal effect on bone collagen synthesis. To more completely understand these effects, studies are under way to define the interrelationship between these molecules and the structural basis for their interaction with the surface of bone-forming and bone-resorbing cells.

TABLE 4. Effect of LPS fractions on the incorporation of [^{14}C]proline into CP and NCP in fetal rat calvaria[a]

Treatment	Concn (μg/ml)	dpm/μg (dry wt)			
		CP		NCP	
		Treated	Control	Treated	Control
Insulin	10^{-8} M	10.3 ± 1.5[b]	6.0 ± 0.7	24.4 ± 2.6	20.3 ± 2.5
S. minnesota LPS	10	5.7 ± 0.7[b]	10.1 ± 1.1	26.7 ± 3.1	28.6 ± 1.9
	1	9.2 ± 1.2	10.9 ± 1.5	29.0 ± 2.7	34.4 ± 2.1
B. gingivalis					
Peak 2	10	6.3 ± 0.6[b]	10.2 ± 0.9	42.9 ± 3.3[b]	58.0 ± 6.8
Peak 3	10	8.8 ± 1.0[b]	12.1 ± 1.4	53.7 ± 5.8	60.7 ± 3.9
	1	7.3 ± 0.9	8.7 ± 1.0	51.4 ± 5.4	54.7 ± 4.8

[a] Values are means ± standard errors of eight calvaria. The calvaria were cut in half along the midsagittal suture and cultured as pairs: one half as experimental, the other as a control. Differences between experimental and control bones were analyzed by a paired *t* test.
[b] Significant difference at $P < 0.05$ level between pairs of data.

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