Serological Properties of Lipopolysaccharide from Oral Strains of *Bacteroides melaninogenicus*

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Lipopolysaccharide (LPS) extracted with phenol-water from four oral strains of *Bacteroides melaninogenicus* was found to be serologically active in precipitation and complement fixation tests and sensitized sheep erythrocytes to agglutination. Except for the capacity to inhibit indirect hemagglutination, the serological activity was destroyed by oxidation with periodate. The isolated LPS was antigenic in rabbits, giving rise to low- and high-molecular-weight antibodies. Cross-reactivity experiments revealed the presence in LPS of both type-specific and group-reactive antigenic determinants.

In recent years, several papers have discussed the chemical, immunological, and biological properties of lipopolysaccharides (LPS) from oral strains of *Veillonella* (1, 11, 12, 13), *Fusobacterium* (1, 11; S. B. Jensen, Ph.D. Thesis, Århus Tandlægehöjskole, Århus, Denmark, 1967), and *Leptotrichia buccalis* (4, 6, 7, 11). In contrast, little is known of the LPS from oral strains of *Bacteroides melaninogenicus*. Biologically and serologically active LPS preparations from this organism have been made by Mergenhagen, Hampp, and Scherp (11) and Courant and Gibbons (2). The preparations, however, were not fully characterized chemically or serologically.

In a previous paper, the chemical composition of LPS extracted with phenol-water from oral strains of *B. melaninogenicus* was reported (8). Quantitative analyses carried out on LPS from two strains revealed the following percentages: neutral sugar, 64 and 57; hexosamine, 5.1 and 15.4; fatty acid ester, 3.3 and 6.6; protein, 5.7 and 10.5; phosphorus, 0.6 and 1.5; and nitrogen, 2.3 and 4.4. Glucose was the predominating sugar. Other sugar components found were rhamnose, xylose, glucosamine, and galactosamine. Heptose and 2-keto-3-deoxy-octonate were not demonstrated.

The present paper presents the results of serological examinations performed with the isolated LPS.

MATERIALS AND METHODS

The *B. melaninogenicus* strains B10, B9, B18, and B22 were isolated from the gingival mucosa of human adults suffering from periodontitis.

Cultivation of strains and preparation of LPS have been described earlier (8).

Treatment of LPS with enzymes and periodate. Enzymatic digestion of LPS was performed with crystalline trypsin (Trypure, Novo, Industri A/S, Copenhagen), protease (Nutritional Biochemicals Corp., Cleveland, Ohio), Pronase (B grade; Calbiochem, Los Angeles, Calif.), papain (Merck & Co., Inc., Rahway, N.J.), and pepsin (three times crystallized; Sigma Chemical Co., St. Louis, Mo.). Treatment with trypsin, protease, and pronase was carried out at pH 7.8 in 0.02 M tris(hydroxymethyl)aminomethane buffer containing 0.05 M Ca++. Digestion with pepsin and papain was performed in 0.1 M citrate buffer at pH 2.0 and at pH 7.0 in 0.1 M phosphate buffer containing 0.01 M cysteine hydrochloride and 0.002 M ethylenediaminetetraacetic acid, respectively. The digestions were carried out at 37 C for 20 hr. Treatment with Pronase was carried out with an enzyme to substrate ratio of 1 to 20 (w/w). All other treatments were performed with a ratio of enzyme to substrate of 1 to 50 (w/w). Immediately after treatment, the mixtures and buffer controls were heated to 100 C for 10 min and dialyzed overnight against the buffers to be used in subsequent serological tests.

Oxidation with periodate was performed by adding 0.2% suspensions of LPS in 0.02 M phosphate buffer, pH 7.0, to equal volumes of 0.05 M sodium *meta*-periodate. The mixtures were incubated in the dark at 4 C for 5 days and were dialyzed against running tap water or 0.07 M phosphate-buffered saline, pH 7.2, before use.

Antisera. Antisera were produced by immunizing rabbits intravenously with a suspension of washed bacteria, standardized to a density of approximately 5 × 10⁹ bacterial cells per ml. Increasing doses were given on 3 successive days each week during a 3-week period. The animals were bled 1 week after the last injection, and the serum was stored at −20 C. Antisera against LPS were prepared in the same way. Each rabbit was given a total dose of LPS of 2.5 or 5.0 mg.

Fractionation of antiserum was carried out on columns of Sephadex G-200 with 0.05 M potassium buffer, pH 7.3, containing 0.4 M NaCl and 0.02% sodium azide as eluant (5).

Treatment of antiserum with 2-mercaptoethanol (ME) was performed by adding equal volumes of a 0.2 M solution of ME in phosphate-buffered saline, pH 7.4, to samples of fractionated serum. The mixtures were kept at room temperature for 1 hr. Thereafter they were dialyzed against 0.02 M iodoacetamide for 6 hr and finally against 0.07 M phosphate-buffered saline, pH 7.2, overnight.

Serological methods. Ring test precipitation was carried out in capillary tubes with undiluted serum. Difco Special Agar (Noble; 1%) in saline was used for gel diffusion tests.

Complement fixation was performed essentially as described by Mayer (10). Two 100% lytic units of complement and 2 units of amboceptor were used. Antigen and inactivated (30 min, 56 C) antiserum were tested for anticomplementary or lytic properties. Tubes with 0.1 ml of serum dilution, 0.1 ml of antigen, and 0.2 ml of complement were incubated overnight at 4 C, after which 0.2 ml of a 1% suspension of sensitized sheep erythrocytes was added. After incubation in a water bath at 37 C for 30 min, the degree of hemolysis was estimated visually.

Sensitization of sheep erythrocytes for indirect hemagglutination was performed by mixing equal volumes of a 0.5% suspension of washed cells and antigen. Sensitization was carried out with untreated LPS or with LPS treated with 0.25 N NaOH for 1 hr at 56 C (9). Unless otherwise stated, 75 μg of LPS in 1 ml was used for sensitization of 1 ml of cell suspension. After incubation at 37 C for 30 min, the sensitized cells were washed three times and resuspended to a 0.5% suspension. Sera to be tested were absorbed with normal sheep erythrocytes, thereafter diluted twofold from 1:10 in 0.2-ml volumes; 0.2 ml of the suspension of sensitized cells was added to each tube. Incubation was performed at room temperature overnight, and the hemagglutination was recorded by reading the patterns. All washings, suspensions, and dilutions were made with 0.07 M phosphate-buffered saline, pH 7.2.

For inhibition of hemagglutination, eight agglutinating doses of antiserum in 0.2-ml volumes were preincubated at 37 C for 30 min with inhibitor in 0.1 ml of buffered saline.

Bacterial agglutination was performed in tubes. Two drops of a saline suspension of boiled bacteria (turbidity corresponding to McFarland's scale no. 10) was added to 0.5 ml of serum in dilution. Agglutination was recorded by reading the patterns after incubation overnight at 37 C.

RESULTS

Serological reactivity. The serological reactivity of LPS from strain B10 (LPS-B10) was examined against antisera to whole B10 bacterial cells.

A 1-mg amount of LPS-B10 per ml was precipitated in the ring test in dilutions as high as 1:32. The precipitating activity varied somewhat

from one batch of LPS to another. In agar double-diffusion tests, one band appeared near the antigen basin after 1 or 2 days at room temperature (see Fig. 2). With some antisera, one or two weak bands were sometimes observed nearer to the serum basin.

Serial dilutions of LPS-B10 were examined by the complement fixation test against serial dilutions of homologous antiserum. Maximal titers, 160 to 640, varying with the serum used, were obtained with dilutions of LPS of from 10 to 100 μg/ml.

The antisera agglutinated sheep erythrocytes sensitized with LPS-B10 in titers varying from 160 to 2,560. The sensitizing activity of LPS-B10 was increased fourfold by treatment with 0.25 N NaOH for 60 min at 56 C (Table 1). The minimal amounts of LPS-B10 needed for inhibition of hemagglutination in eight hemagglutinating doses of antiserum varied with the batches used between 0.1 and 0.05 μg, and was the same for untreated and NaOH-treated samples.

LPS prepared from the *B. melaninogenicus* strains B9, B18, and B22 behaved principally as LPS-B10 in ring test precipitation and complement-fixation tests when examined against homologous antisera. However, untreated LPS from strains B18 and B22 did not sensitize sheep red blood cells to agglutination in homologous antiserum (Table 1). After treatment with 0.25 N NaOH, sensitization was obtained with LPS-B18 and a single batch of LPS-B22.

Treatment of LPS with proteolytic enzymes and periodate. The precipitating activity of LPS-B10 was unaffected by digestion with proteolytic enzymes. In contrast, no ring test precipitation or agar precipitation lines were observed after oxidation with periodate.

The effects of treatment with proteolytic enzymes and periodate on the erythrocyte-sensitizing, complement-binding, and antibody-neutralizing capacities of LPS-B10 are compiled in Table 2. NaOH-treated LPS was used for examination of the effects on its erythrocyte-sensitizing power. As shown in Table 2, no complement binding or erythrocyte sensitization was achieved after periodate oxidation with doses of LPS-B10 as high as 2 or 250 μg/ml, respectively. However, the antibody-neutralizing capacity, as measured by inhibition of hemagglutination, was only reduced.

Antigenicity. Antisera from two rabbits immunized with LPS-B10 contained precipitating and complement-binding antibodies to LPS-B10 (titer, 80) and agglutinated sheep-cells sensitized with LPS-B10 (titer, 2,560). The antisera agglutinated boiled B10 bacterial cells at a titer of 80.

TABLE 1. *Erythrocyte-sensitizing activities of LPS from B. melaninogenicus*

LPS	Sensitizing activity (μg/ml)[a]				Titer, indirect hemagglutination[c]
	Untreated		NaOH-treated[b]		
	Detectable agglutination	Maximal agglutination	Detectable agglutination	Maximal agglutination	
LPS-B10..............	25	100	6.25	25	160–2,560
LPS-B9...............	12.5	50	12.50	50	2,560
LPS-B18..............	>200	>200	6.25	25	80
LPS-B22..............	>200	>200	6.25	25	1,280

[a] Concentration of LPS required for sensitization of an equal volume of a 0.5% erythrocyte suspension.

[b] Treatment: 0.25 N NaOH, 60 min at 56 C.

[c] Reciprocal of maximal titer, homologous antiserum.

TABLE 2. *Effect of proteolytic enzymes and periodate on the erythrocyte-sensitizing, complement-binding, and antibody-neutralizing capacity of LPS from B. melaninogenicus strain B10*

Treatment	Sensitizing activity (μg/ml)[a]	Complement-binding activity (μg)[b]	Minimal inhibiting dose (μg)[c]
Pronase, protease, pepsin, papain, trypsin.............	25–50	0.2–0.4	0.1
Buffer...............	50	0.2–0.4	0.1
Periodate...........	>250	>2	1.6
Buffer...............	25	0.2	0.2

[a] Least concentration of LPS-B10 added to an equal volume of 0.5% erythrocyte suspension necessary to give maximum serum titers.

[b] Least amount of LPS-B10 binding two 100% lytic units of complement.

[c] Indirect hemagglutination, 8 agglutinating units of antiserum.

Both antisera were fractionated on columns of Sephadex G-200. In the experiment outlined in Fig. 1, 1 ml of undiluted serum was applied on a column (45 by 2.5 cm). Elution was performed at room temperature with a flow rate of 20 ml/hr. Fractions of 5 ml were collected and examined for complement-binding antibodies and for antibodies agglutinating sheep cells sensitized with LPS-B10. The complement-binding and erythrocyte-modifying antibodies were associated with the first peak containing mainly immunoglobulin M (IgM) and also with the second peak containing mainly immunoglobulin G. ME treatment of the fractions completely destroyed all antibody activity in the fractions corresponding to the first peak (fractions 14 to 19), whereas the fractions
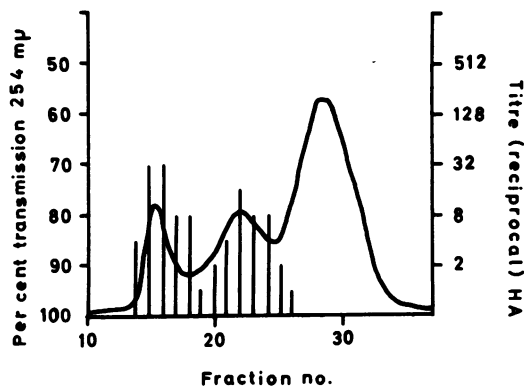

FIG. 1. *Fractionation of 1 ml of antiserum LPS-B10 on Sephadex G-200. The bars represent titer values in indirect hemagglutination (HA) of the different fractions. Fractions of 5 ml were collected.*

corresponding to the second peak (fractions 20 to 26) reacted to the same titers as before.

Cross-reactivity. LPS preparations from strains B10, B9, B18, and B22 were examined for cross-reactivity by ring test precipitation, complement fixation, indirect hemagglutination, and by double diffusion in agar. The antisera used were produced against whole bacterial cells.

All LPS preparations cross-reacted in each other's antisera. The precipitating activity of LPS in heterologous antisera was very weak with some preparations. In agar double-diffusion tests, the principal bands given by the four LPS preparations showed a reaction of nonidentity when mixtures of two and two LPS preparations were examined against their antisera. This is exemplified by the schematic drawing shown in Fig. 2. The results of complement fixation tests are shown in Table 3.

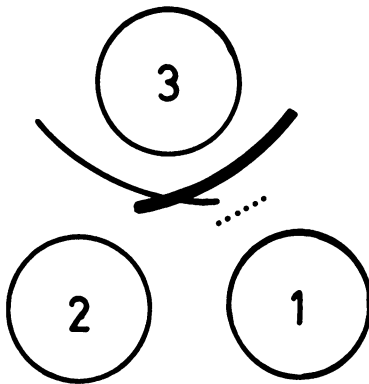All antisera contained antibodies reacting with

FIG. 2. Schematic drawing of the agar precipitation bands produced by LPS-B10 and LPS-B22 against homologous antiserum. Well 1, antiserum B22; well 2, antiserum B10; well 3, equal volumes of LPS-B10 and LPS-B22 (2 mg/ml).

TABLE 3. Complement-binding antibodies to B. melaninogenicus LPS in homologous and heterologous antisera

LPS	Titer (reciprocal) in antiserum			
	B10	B9	B18	B22
LPS-B10..........	320	160	160	320
LPS-B9...........	320	320	320	160
LPS-B18..........	40	160	160	80
LPS-B22..........	10	40	20	1,280

sensitized erythrocytes, and the titers were similar irrespective of the LPS preparation used for sensitization. This indication of a shared erythrocyte-sensitizing antigen, to which antibodies had been produced in high titers (160 to 2,560), was confirmed by hemagglutination inhibition experiments. The agglutination of LPS-B10-sensitized erythrocytes in B10 antiserum was completely inhibited by small doses of homologous and heterologous LPS (Table 4).

DISCUSSION

B. melaninogenicus LPS preparations were serologically active in the tests employed, particularly in respect to complement-binding and antibody-neutralizing capacity. Similar to LPS from aerobic bacilli (3) and oral strains of *Veillonella* (13), the ability to sensitize erythrocytes to agglutination could be enhanced or was disclosed by treatment with alkali. The LPS preparation from strain B9 was an exception. However, relatively large doses of LPS had to be used to obtain sensitization. As the opaque suspension of LPS did not clear completely during treatment

TABLE 4. Antibody-neutralizing capacity of B. melaninogenicus LPS preparations[a]

LPS	Minimal inhibiting dose (μg)[b]
LPS-B10.............................	0.05
LPS-B22.............................	0.8
LPS-B9..............................	0.05
LPS-B18.............................	0.006

[a] Measured by inhibition of agglutination of erythrocytes sensitized with LPS from *B. melaninogenicus* strain B10, in B10 antiserum.

[b] Eight agglutinating units of antiserum.

with NaOH, it is possible that the concentration of NaOH used was not optimal for this particular LPS.

The results attained by periodate oxidation suggest that the polysaccharide moiety of the LPS complex is essential for its serological activity in precipitation and complement-fixation tests and for the erythrocyte-modifying ability. In this respect, *B. melaninogenicus* LPS preparations behave like LPS from aerobic bacteria. However, the antibody-neutralizing capacity, as measured by inhibition of hemagglutination, was only slightly affected. Apparently, the antigenic determinant (or determinants) reacting with antibodies to the sensitized sheep cells is relatively resistant to treatment with periodate or, less likely, is in some way protected during oxidation. The insensitivity to proteolytic enzymes makes it unlikely that the small protein moiety is responsible for part of the serological activity.

The LPS extracted with phenol-water behaved as a fairly good antigen, giving rise to serum titers comparable to those attained when whole bacterial cells were used for immunization. Most of the antibodies specific for LPS of aerobic gram-negative bacilli are commonly found in the IgM class of immunoglobulins (16). In the present study, low-molecular-weight antibodies were also demonstrated in rabbit antisera, but the high-molecular-weight antibodies were predominant (according to activity). Similar findings have been reported by Weidanz, Jackson, and Landy (17) and by Pike and Shulze (15), working with LPS (somatic antigen) from *Salmonella* and *Escherichia coli*, and may in part be due to the massive amounts of antigen used for immunization (14). They did not investigate, however, whether antibodies belonging to the immunoglobulin A class of immunoglobulins had been produced.

The occurrence of nonidentical agar precipitation bands and the weak precipitating activity in some heterologous sera, together with the varying titers obtained by complement fixation, suggest

that LPS from the strains B10, B9, B18, and B22 contain type-specific antigenic determinants. This is not an unexpected finding as type specificity in gram-negative bacteria, among them oral strains of *Veillonella* (12), *L. buccalis*, and *Fusobacterium* (4), is a function of the polysaccharide within the LPS complex. Finding cross-reactivity was interesting, particularly in view of the recent demonstration in human sera of antibodies to sheep erythrocytes sensitized with *B. melaninogenicus* LPS (2), a discovery which has been corroborated in this laboratory. The inhibitory effect of heterologous LPS preparations on the agglutination of LPS-B10-modified sheep cells in B10 antiserum indicates that LPS from oral strains of *B. melaninogenicus* possess one or more group-specific antigenic determinants.

The hemagglutination inhibition experiments do not exclude the existence, in B10 antiserum, of type-specific antibodies to LPS-B10-modified erythrocytes. If they have low titers such antibodies have been missed, as only eight agglutinating doses of antiserum were used in these experiments.

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