JOURNAL OF BACTERIOLOGY, Dec., 1965
Vol. 90, No. 6

Polysaccharide-Lipid Complexes from *Veillonella parvula*

STEPHAN E. MERGENHAGEN

Laboratory of Microbiology, National Institute of Dental Research, U.S. Public Health Service, Bethesda, Maryland

Received for publication 9 August 1965

ABSTRACT

MERGENHAGEN, STEPHAN E. (National Institutes of Health, Bethesda, Md.). Polysaccharide-lipid complexes from *Veillonella parvula*. J. Bacteriol. **90**:1730–1734. 1965.—A strain of *Veillonella parvula* (V2) elaborates an extracellular slime when grown in a nutrient medium containing only dialyzable components. Deproteinization with chloroform-butanol of ethyl alcohol-precipitated material from the supernatant culture fluid leads to the isolation of a water-soluble lipopolysaccharide (LPS1). Another component (LPS2)), showing similarity in biological and immunological properties to the endotoxic antigen (LPC) isolated from whole cells, was extracted with phenol from the insoluble emulsion remaining after chloroform-butanol extraction of slime. Analysis of polysaccharides by thin-layer chromatography demonstrated the presence of glucose and galactose in LPS1 and glucose, glucosamine, galactosamine, and a methyl pentose in LPC. LPS1 failed to give a positive epinephrine skin test after intravenous injection in rabbits and failed to kill pertussis-sensitized mice, whereas LPS2 and LPC were active in both of these bioassays. Both lipopolysaccharides (LPS1 and LPC) exhibited type-specific haptenic activity in hemagglutination tests with numerous anti-*Veillonella* rabbit sera. LPS1 was found in these tests to be unrelated to a heterologous strain of *Veillonella* possessing a related somatic antigen. These experiments reveal the presence of two chemically and immunologically distinguishable polysaccharide-lipid complexes in this strain of *V. parvula*.

It is now possible to define serotypes of human oral strains of gram-negative anaerobic bacteria belonging to the genera *Veillonella*, *Fusobacterium*, and *Leptotrichia* by utilizing their endotoxic somatic lipopolysaccharides in hemagglutination-inhibition tests (Mergenhagen and Varah, 1963; de Araujo, Varah, and Mergenhagen, 1963). Previous studies have revealed the unsuspected antigenic individuality of the oral gram-negative microbiota in different individuals.

Further work in this area was undertaken when it was found that an oral strain of *V. parvula*, which has been used in our laboratory for some time, elaborates an extracellular slime. From this slime, a serologically specific lipopolysaccharide has been obtained which is immunologically and chemically distinguishable from the endotoxic somatic lipopolysaccharide of the same organism. It is also serologically unrelated to another strain of *Veillonella* possessing a related somatic antigen.

MATERIALS AND METHODS

Isolation of polysaccharide-lipid complex from extracellular slime. V. parvula strain V2 was cultured for 18 to 24 hr at 37 C in 17 liters of the dialyzable components of a Trypticase-yeast extract medium to which were added sodium lactate and sodium thioglycolate. For each 3 liters of medium, 15 g of Trypticase and 12 g of yeast extract were dissolved in 100 ml of distilled water and placed in a dialysis membrane. This was then dialyzed overnight against 3 liters of distilled water. To the diffusate were added 20.8 ml of sodium lactate per liter and 0.75 g of sodium thioglycolate per liter. The medium was then sterilized by autoclaving.

Cells were removed from the culture medium by centrifugation at 10,000 rev/min. To each volume of supernatant fluid, 2 volumes of 95% ethyl alcohol were added. The stringy, viscous precipitate which formed immediately was easily removed from the surface with a glass rod. The total precipitate collected was centrifuged and washed in ethyl alcohol; finally dispersed in distilled water, producing a highly viscous solution; and then dialyzed exhaustively against distilled water. The material was then lyophilized and deproteinized. To 700 mg were added 200 ml of distilled water, 20 g of sodium acetate, 2 ml of glacial acetic acid, 40 ml of chloroform, and 8 ml of *n*-butanol. The contents were mixed in the cold (5 C) in a Waring Blendor for 90 min, followed by centrifugation at 2,000 rev/min for 30 min. The water phase was

withdrawn and stored at 5 C. To the remaining emulsion, 200 ml of distilled water were added. The contents were shaken in a flask overnight at 5 C. The water phase was collected after centrifugation and was pooled with the first water phase. The pooled water phases were treated twice with butanol and chloroform as before, and were finally separated by centrifugation. To the water phase were added 600 ml of 95% ethyl alcohol and a small quantity (50 to 100 mg) of sodium chloride. The precipitate which formed overnight at 5 C was collected by centrifugation, dissolved in distilled water, and lyophilized (designated as LPS1). Such material weighed approximately 151 mg or represented 22% of the starting material.

In addition, the emulsion layers which formed after chloroform-butanol extraction of slime were pooled and centrifuged. The sediment was washed, suspended in distilled water, dialyzed, and finally lyophilized. The material was then extracted with 100 ml of phenol and 100 ml of distilled water for 15 min in a Waring Blendor. The water phase was removed, exhaustively dialyzed against distilled water, and centrifuged in a Spinco model L centrifuge for 2 hr at 40,000 rev/min. The sediments were pooled, dispersed in distilled water, and lyophilized (designated as LPS2).

Isolation of somatic lipopolysaccharide. The somatic lipopolysaccharide from *V. parvula* was isolated by phenol-water extraction of washed whole cells (de Araujo et al., 1963). The water phase was exhaustively dialyzed against distilled water, and the somatic lipopolysaccharide was isolated by centrifugation at 40,000 rev/min for 2 hr. Sediments were pooled, solubilized in distilled water, and lyophilized (designated as LPC). The somatic lipopolysaccharide from a heterologous strain of *V. parvula* (BYR-2) was isolated from washed whole cells from mass culture in an identical manner.

Preparation of "crude" group antigen. Soluble antigens from strain V2 were prepared from washed cells in a French pressure cell (Aminco) and were used in hemagglutination tests in a similar manner to that previously described (de Araujo et al., 1963). Cell debris was removed by centrifugation, and a 1:10 dilution of the supernatant fluid ("crude" antigen) was used to sensitize a standard suspension of sheep erythrocytes.

Antisera. Rabbit antiserum to *V. parvula* V2 was prepared by an intensive schedule of intravenous injections of viable, washed cells. Other rabbit antisera to various heterologous strains of human oral veillonellae were kindly supplied by Morrison Rogosa, National Institute of Dental Research. Hemagglutination tests and Ouchterlony analyses were performed by methods already described (de Araujo et al., 1963).

Chemical analysis of lipopolysaccharides. For analysis of polysaccharides by thin-layer chromatography, 10 mg of product were hydrolyzed in 1.0 ml of 1 N HCl in a sealed ampoule for 3 hr at 100 C. The contents were deionized by batch treatment with Dowex-1-X8 generated with NaOH. The resin was removed by centrifugation and 0.02 ml of the hydrolysate was applied to a cellulose plate. A mixture of reference sugars was included on every chromatogram. Ascending thin-layer chromatograms were run in butanol-pyridine-water (9:5:8), and were developed with silver nitrate reagent. To verify the presence of methyl pentose, a corresponding undeveloped area along with control areas was scraped off the glass plate, eluted in hot distilled water, and assayed spectrophotometrically (Dische and Shettles, 1948). To verify the presence of hexosamines, hydrolysates were also run on Whatman no. 1 paper and were developed with a hexosamine spray reagent (Block, Durrum, and Zweig, 1955). Total carbohydrate in the product was determined by the anthrone method (Shields and Burnett, 1960), and protein was estimated by the procedure of Lowry et al. (1951). Total bound lipid content of the product was determined by a method previously described (Mergenhagen, Martin, and Schiffmann, 1963).

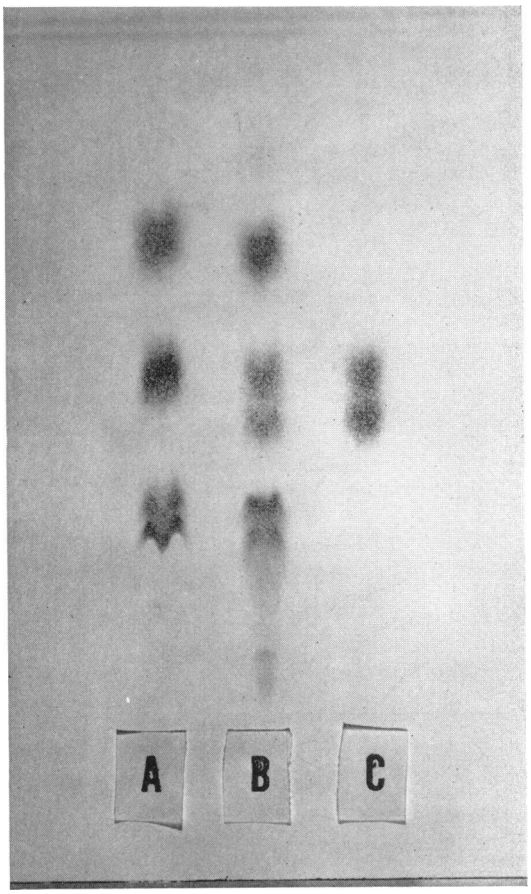

FIG. 1. *Thin-layer chromatographic analysis of hydrolysates of Veillonella polysaccharides. (A) Somatic lipopolysaccharide (LPC). (B) Standards in ascending order: glucosamine, galactosamine galactose, glucose, and fucose. (C) Extracellular lipopolysaccharide (LPS1) from slime.*

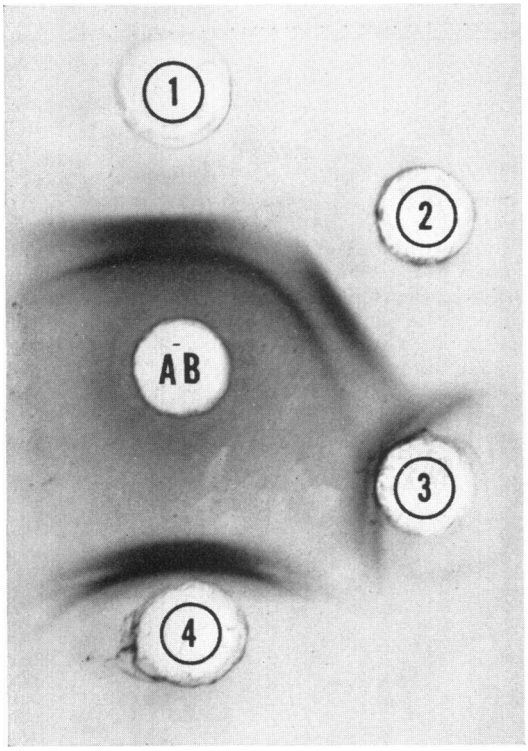

FIG 2. *Ouchterlony analysis of lipopolysaccharide fractions from Veillonella. High-titered rabbit antiserum to Veillonella in center well (AB); (1) extracellular lipopolysaccharide LPS1 from slime (1,000 μg/ml); (2) extracellular lipopolysaccharide LPS1 from slime (500 μg/ml); (3) LPS2 from slime (1,000 μg/ml); (4) somatic lipopolysaccharide LPC (1,000 μg/ml). Precipitates stained with 0.1% Naphthol blue-black (obtained from Allied Chemical Corp., Morristown, N.J.).*

Tests for biological activity. Lyophilized products of *Veillonella* were dissolved in 0.85% sodium chloride and were tested for lethality in female NIH mice (16 to 18 g) after 7 days of sensitization by intraperitoneal injection of 0.2 ml of *Bordetella pertussis* vaccine (Eli Lilly & Co., Indianapolis, Ind.). The epinephrine skin tests were performed as previously described (de Araujo et al., 1963).

RESULTS AND DISCUSSION

The preparation of water-soluble lipopolysaccharide (LPS1) isolated after deproteinization of extracellular slime contained 52% carbohydrate and 24% bound lipid, whereas the somatic lipopolysaccharide (LPC) consisted of 18% carbohydrate by Anthrone analysis and 52% lipid. Both preparations contained less than 3% protein. LPS1 dispersed very easily in aqueous media yielding clear solutions; LPC was only sparingly soluble in water, as is the case with other somatic lipopolysaccharides isolated from gram-negative bacteria by phenol-water extraction (Ribi et al., 1962; Mergenhagen and Martin, 1964).

Analysis of acid hydrolysates of each of these preparations by thin-layer chromatography revealed that the somatic lipopolysaccharide (LPC) contained glucosamine, galactosamine, glucose, and a fast-moving component comparable to the methyl pentose, fucose (Fig. 1). The fact that this material contains a good proportion of amino sugars accounts for the low total carbohydrate as estimated by the anthrone test. On the other hand, the LPS1 fraction, isolated from extracellular slime, was found to be composed solely of the monosaccharides glucose and galactose (Fig. 1). It has been found that this extracellular *Veillonella* lipopolysaccharide (LPS1) gives strong precipitin reactions with type XIII and XX antipneumococcal sera (M. Heidelberger, *personal communication*). The polysaccharides of these pneumococcal types contain glucose, galactose, and glucosamine (Shabarova, Buchanan, and Baddiley, 1962).

Ouchterlony tests, with anti-*Veillonella* rabbit serum, showed that LPS1 was composed of two fast-diffusing components, whereas the somatic

TABLE 1. *Agglutination of sheep erythrocytes sensitized with antigens from Veillonella parvula V2 by homologous and heterologous anti-Veillonella rabbit sera*

Antigen from strain V2 on erythrocytes	Antisera to various strains of *Veillonella*					
	V2	PRI	BYR-2	N4282	BL78	ERN
"Crude" antigen	10,240*	160	1,280	640	80	640
LPS1† (extracellular water-soluble lipopolysaccharide)	2,560	<40	80	<40	<40	<40
LPC† (somatic lipopolysaccharide)	5,120	<40	2,560	<40	<40	<40

* Hemagglutination titer (reciprocal).

† Both lipopolysaccharides were treated with 0.02 N NaOH for 18 to 24 hr at 37 C before modifying sheep erythrocytes for hemagglutination tests.

TABLE 2. *Inhibition of the agglutination of sheep erythrocytes sensitized with lipopolysaccharide by lipopolysaccharide-containing fractions from Veillonella parvula V2*

Fraction tested for inhibition	Highest dilution (reciprocal) of 0.1% solution of tested fraction inhibiting the hemagglutination reaction*	
	LPS1-sensitized erythrocytes†	LPC-sensitized erythrocytes†
LPS1	2,048	32
LPS2	64	1,024
LPC	32	1,024

* Inhibition test with 1:1,000 dilution of anti-V2 rabbit serum.

† Lipopolysaccharide treated with 0.02 N NaOH for 18 to 24 hr at 37 C before modifying sheep erythrocytes.

lipopolysaccharide (LPC) was composed of multiple, slow-diffusing components (Fig. 2). It is interesting to note that the fraction (LPS2) isolated by phenol extraction from the insoluble emulsion formed after deproteinization of slime contained slow-diffusing, multiple components. Taken in conjunction with the bioassays and hemagglutination-inhibition tests on these materials which will be presented shortly, the presence of endotoxic somatic lipopolysaccharide in the extracellular slime is indicated.

Employing rabbit antisera to several human oral strains of veillonellae in hemagglutination tests demonstrated that sheep erythrocytes sensitized with "crude" antigen from strain V2 were agglutinated by homologous as well as several heterologous antisera, indicating that these organisms share at least one antigenic component (Table 1). The somatic lipopolysaccharide-coated erythrocytes were agglutinated by homologous antiserum and by one heterologous antiserum, indicating a serological relationship between the O antigens of strains V2 and BYR-2. By Ouchterlony analysis, lipopolysaccharides from strains V2 and BYR-2 showed comparable migration rates in gel when they were precipitated in gel with anti-V2 rabbit serum. In biochemical tests for taxonomic purposes, strain BYR-2 is identical to strain V2 (M. Rogosa, *personal communication*). In contrast to strain V2, however, strain BYR-2 does not produce an extracellular slime. Interestingly enough, extracellular lipopolysaccharide (LPS1) from strain V2 is serologically type-specific, since erythrocytes coated with this hapten were agglutinated to a high titer with homologous antiserum only. The low titer observed with anti-BYR-2 rabbit serum and LPS1 is possibly indicative of some contamination of LPS1 with somatic lipopolytaccharide or cross-reaction between polysaccharide components. The fact that well-washed cells of strain V2 induce antibodies in the rabbit to this extracellular lipopolysaccharide may indicate that this antigen is attached as a capsulelike substance around the cell wall of the organism.

Table 2 illustrates that, at a high concentration, the somatic lipopolysaccharide (LPC) inhibits antibody to LPS1. A similar situation exists with regard to the inhibition of antibody to LPC by LPS1. These tests indicate a contamination or a cross-reaction of these lipopolysaccharides which could not be detected by Ouchterlony analysis or by the biological testing which will be presented subsequently. These tests further show that LPS2, suspected of being related to the somatic lipopolysaccharide, has a similar serological reactivity.

Tests for biological activity indicate that endotoxicity is associated with phenol-water preparations from whole cells (LPC) and similar preparations (LPS2) from the insoluble emulsion after deproteinization of slime but not with the water-soluble LPS1 after deproteinization of slime (Table 3).

From this work and that of others (Srivastava et al., 1962), it is apparent that certain gram-negative organisms produce more than one polysaccharide-lipid complex, either in their cell-wall components or extracellularly. The obvious interesting difference between our findings and those of others is the lack of biological activity characteristic of endotoxins in the water-soluble lipopolysaccharide isolated from the extracellular slime elaborated by *Veillonella*. The apparent difference in chemical composition, serological reactivity, and physical properties of the extracellular lipopolysaccharide and the somatic endotoxic lipopolysaccharide of the same organism may eventually provide a clue as to the chemical and physical properties necessary for

TABLE 3. *Lethal activity in mice and dermal reactivity in rabbits of Veillonella parvula V2 lipopolysaccharide-containing fractions*

Prepn	Lethality in pertussis-sensitized mice after intraperitoneal injection of (μg)					Dermal reactivity of rabbits to epinephrine after intravenous injection of (μg)			
	400	200	100	50	25	50	10	5	1.0
LPS1	1/6*	0/6	0/6	0/6	0/6	0/3†	0/3	0/3	0/3
LPS2	6/6	5/6	5/6	2/6	0/6	2/3	1/3	1/3	0/3
LPC	6/6	3/6	3/6	0/6	1/6	2/3	1/3	2/3	0/3

* Dead/total injected after 48 hr.

† Number of rabbits positive for dermal hemorrhage and necrosis/total injected 18 hr after intradermal injection of 100 μg of epinephrine.

those biological properties characteristic of endotoxins.

ACKNOWLEDGMENTS

I would like to thank Elliott Schiffmann for lipid analyses on the polysaccharide-lipid complexes from *Veillonella* and Eileen Watts for technical assistance.

LITERATURE CITED

DE ARAUJO, W. C., E. VARAH, AND S. E. MERGENHAGEN. 1963. Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia*. J. Bacteriol. **86**:837–844.

BLOCK, R. J., E. L. DURRUM, AND G. ZWEIG. 1955. A manual of paper chromatography and paper electrophoresis, p. 152. Academic Press, Inc., New York.

DISCHE, Z., AND L. B. SHETTLES. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

MERGENHAGEN, S. E., AND G. R. MARTIN. 1964. Properties of a lysozyme-dissociated endotoxic fraction from *Escherichia coli*. J. Bacteriol. **88**: 1169–1174.

MERGENHAGEN, S. E., G. R. MARTIN, AND E. SCHIFFMANN. 1963. Studies on an endotoxin of a group C *Neisseria meningitidis*. J. Immunol. **90**: 312–317.

MERGENHAGEN, S. E., AND E. VARAH. 1963. Serologically specific lipopolysaccharides from oral *Veillonella*. Arch. Oral Biol. **8**:31–36.

RIBI, E., W. T. HASKINS, K. C. MILNER, R. L. ANACKER, D. B. RITTER, G. GOODE, R.-J. TRAPANI, AND M. LANDY. 1962. Physiochemical changes in endotoxin associated with loss of biological potency. J. Bacteriol. **84**:803–814.

SHABAROVA, Z. A., J. G. BUCHANAN, AND J. BADDILEY. 1962. The composition of pneumococcus type-specific substances containing phosphorus. Biochim. Biophys. Acta **57**:146–148.

SHIELDS, R., AND W. BURNETT. 1960. Determination of protein-bound carbohydrate in serum by a modified anthrone method. Anal. Chem. **32**: 885–886.

SRIVASTAVA, H. C., E. BRENNINGER, H. J. CREECH, AND G. A. ADAMS. 1962. Preparation and properties of polysaccharide-lipid complexes from *Serratia marcescens*. Can. J. Biochem. Physiol. **40**:905–918.