IMMUNOCHEMICAL ANALYSIS OF HUMAN ORAL STRAINS OF *FUSOBACTERIUM* AND *LEPTOTRICHIA*

WILSON C. DE ARAUJO[1], EILEEN VARAH, AND STEPHAN E. MERGENHAGEN

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

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ABSTRACT

DE ARAUJO, WILSON C. (National Institute of Dental Research, Bethesda, Md.), EILEEN VARAH, AND STEPHAN E. MERGENHAGEN. Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia*. J. Bacteriol. **86**:837–844. 1963.—Lipopolysaccharides, isolated by phenol-water extraction of 27 strains of oral gram-negative bacteria conforming either to *Fusobacterium polymorphum* or *Leptotrichia buccalis*, were shown to be endotoxic by their ability to alter dermal reactivity to epinephrine and to be serologically specific by hemagglutination and hemagglutination-inhibition tests. Numerous serotypes of these organisms were detected by hemagglutination tests with purified lipopolysaccharides. A *F. polymorphum* lipopolysaccharide produced two visible precipitin bands in agar gel with antiserum prepared against the homologous organism. Each of the immunologically distinct components of the endotoxin, isolated by differential centrifugation, altered dermal reactivity to epinephrine and acted as a hapten in hemagglutination tests. Crude antigens from *F. polymorphum* strains, released in supernatant fluids of heat-killed bacterial suspensions, showed broad serological cross-reactivity with antiserum prepared against homologous and heterologous strains of *F. polymorphum* but not with antiserum prepared against *L. buccalis* strains. Broad serological cross-reactivity of these crude *F. polymorphum* antigens could be eliminated by prior treatment with phenol or trypsin, indicating that the common antigen or antigens in these organisms are protein. Double-diffusion tests in agar identified and differentiated type-specific lipopolysaccharide from other antigens extracted by heat from these organisms. Similarly prepared crude antigens from *L. buccalis* had broad serological activity with antiserum prepared against various strains of *L. buccalis* but not with *F. polymorphum*. In contrast to the crude antigens from *F. polymorphum*, this serological cross-reactivity could not be eliminated by treatment with phenol or trypsin.

Little is known with certainty about the antigenic structure of presumably related strains of the genera *Fusobacterium* and *Leptotrichia* (Omata and Braunberg, 1960; Gilmour, Howell, and Bibby, 1961) indigenous to the human oral cavity. Numerous investigations have attempted to establish serological groups or types of these bacteria (Pratt, 1927; Varney, 1927; Slanetz and Rettger, 1933; Spaulding and Rettger, 1937; Brocard, 1939; Bøe, 1941); however, these studies were of little value, since these bacteria agglutinated spontaneously in conventional agglutination tests used. Type-specific proteins and a group-specific carbohydrate were reported in *Fusobacterium* by Weiss and Mercado (1938), who used precipitin tests with cell extracts from four strains. Recently, Gustafson and Kroeger (1962) constructed a tentative picture of the antigenic complex of *L. buccalis* (*F. fusiforme*). They proposed that the outermost layer of the cell contains a type-specific antigen, while several antigens which are shared by several strains of the organism may be contained in the cytoplasm.

The O antigens (endotoxins and lipopolysaccharides) of gram-negative bacteria generally are composed of polysaccharide, lipid, and a small amount of protein. The polysaccharide moiety endows the antigen with serological specificity. In recent years, several methods have become available for the isolation and purification of lipopolysaccharide endotoxins retaining antigenic properties. In recent studies utilizing the phenol-water extraction procedure of Westphal and Lüderitz (1954), it was reported that the endotoxin of oral anaerobic gram-negative cocci (genus *Veillonella*), similar to the somatic O

[1] Visiting Fellow, National Institute of Dental Research, 1962–63. Present address: Faculdade Nacional de Odontologia, Rio de Janeiro, Brazil.

antigens of other bacteria, accounted in major part for the specific agglutinating properties of the organism in homologous antiserum (Mergenhagen, Zipkin, and Varah, 1962). Furthermore, type-specific lipopolysaccharides were detected in several strains of the genus *Veillonella* by utilizing the indirect bacterial hemagglutination test (Mergenhagen and Varah, 1963).

The present investigation was carried out (i) to characterize immunochemically purified lipopolysaccharide endotoxins extracted from strains of *F. polymorphum* and *L. buccalis*, (ii) to ascertain by hemagglutination and hemagglutination-inhibition tests whether such purified lipopolysaccharides determine serological specificity of these organisms as they do in other oral gram-negative bacteria, and (iii) to identify other antigens of these bacteria and ascertain by indirect bacterial hemagglutination whether they react with any serological specificity.

MATERIALS AND METHODS

Cultures. A total of 22 strains of *F. polymorphum* were isolated from the oral cavities of 12 different individuals. *F. polymorphum* ATCC 10953 was obtained from the American Type Culture Collection. Four strains of *L. buccalis* (C1100, C1013b, C1327, and C1170) were obtained from Arden Howell, Jr., National Institute of Dental Research. Two strains of *L. buccalis* (CA3 and CA5) were freshly isolated from human oral material on the medium of Baird-Parker (1957) without the additions of ethyl violet or neomycin sulfate. Biochemical tests (indole and hydrogen sulfide production, gelatin liquefaction, nitrate reduction, presence of catalase, and fermentation of glucose, maltose, mannitol, lactose, sucrose, galactose, fructose, inositol, and raffinose) were performed on all strains to characterize them as *F. polymorphum* or *L. buccalis*. The general procedures were taken from the Society of American Bacteriologists (1957) *Manual of Microbiological Methods*. These biochemical tests were carried out in the basal medium described by Omata and Disraely (1956) for oral fusobacteria and that described by Thjøtta, Hartmann, and Bøe (1939) for *L. buccalis*. Brain Heart Infusion (Difco) supplemented with 0.2% yeast extract (Difco) was used for mass culture of the organisms.

Preparation of antisera. The organisms were grown in 20-ml portions of media for 2 days at 37 C. Cells were collected by centrifugation, washed in distilled water, and resuspended in 5 ml of 0.85% sodium chloride. New Zealand albino rabbits, weighing 2.5 to 3.0 kg, were immunized by intravenous injection of 1.0 ml of fresh bacterial suspension on 3 consecutive days, followed by a booster of 1.0 ml 5 days after the last injection. Blood was withdrawn 5 days after the booster by cardiac puncture; the serum was collected and stored at −20 C.

Preparation of "crude antigen." Cells from 3-liter batches of media were harvested by centrifugation, suspended in 50 ml of 0.5 M phosphate buffer (pH 7.3), and heated at 100 C for 1 hr. Cell debris was removed by centrifugation at 13,000 × *g*, and a 1:10 dilution of the supernatant fluid ("crude antigen") was used to sensitize sheep erythrocytes for indirect bacterial hemagglutination tests. In certain cases, liquid phenol (90% aqueous) was added to an equal volume of the "crude antigen," and the mixture was homogenized in a Waring Blendor for 30 min. The phenol phase was separated by centrifugation, and the upper water phase was removed and dialyzed against 0.5 M phosphate buffer for 48 hr. In other experiments, 2 mg of crystalline trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio) were added to 100 mg of lyophilized "crude antigen" in 10 ml of 0.5 M phosphate buffer (pH 7.3). This mixture was incubated for 48 hr at 37 C, and then was heated at 80 C for 1 hr to inactivate the enzyme. This material was then dialyzed against 0.5 M phosphate buffer for 48 hr.

Preparation of purified lipopolysaccharide endotoxin. Lipopolysaccharide was prepared in a manner similar to that of Westphal and Lüderitz (1954). Cells from 12-liter batches of media were harvested by centrifugation, washed in distilled water, and resuspended in 110 ml of distilled water. An equal volume of liquid phenol (90% aqueous) was added, and the contents were stirred in a Waring Blendor for 15 min. The water phase was separated by centrifugation and dialyzed against running tap water to remove phenol. The dialyzed water phase was centrifuged at 100,000 × *g* for 1 hr. The gelatinous deposits were resolubilized in distilled water and lyophilized ("purified lipopolysaccharide"). Further separation of endotoxin by differential centrifugation into two immunologically distinct components was carried out by the technique of Ribi et al. (1962).

Hemagglutination tests. These tests were per-

formed as described previously (Mergenhagen et al., 1962). Sheep erythrocytes (Cappel Laboratory, West Chester, Pa.) were washed twice as a 0.5% suspension. The antigen was added in proper dilution to the sedimented erythrocytes to make a 0.5% erythrocyte suspension. The mixtures were incubated for 2 hr at 37 C. The erythrocytes were washed twice to remove excess antigen. Portions (0.5 ml) of the erythrocyte suspension were added to 0.5-ml portions of serial dilutions of antiserum. The mixtures were incubated for 2 hr at 37 C, refrigerated overnight, and read the next morning. In hemagglutination-inhibition tests, 0.5-ml portions of antigen were added to 0.5-ml portions of serial dilutions of antiserum. After mixtures had incubated for 1 hr at 37 C, sensitized erythrocytes were added to each tube. The tubes were incubated as previously described and read the next morning.

Agar gel diffusion. The technique of Ellner and Bohan (1962) was used. Briefly, 0.45 g of Ionagar #2 (Oxoid) was dissolved in 100 ml of 0.85% sodium chloride containing 0.01% phenol. The melted agar was clarified by filtration through Whatman no. 40 paper and was dispensed in glass petri plates. Preliminary tests to determine the optimal concentration of lipopolysaccharide and antiserum indicated that concentrations of antigen ranging from 1 to 5 mg with undiluted antiserum produced clearly visible precipitin bands. "Crude antigen," after lyophilization, was used in concentrations of 2 to 10 mg per ml with undiluted antiserum.

Biological tests for endotoxic activity. The epinephrine skin test (EST) was used for bioassay of lipopolysaccharides. These tests were performed with purified lipopolysaccharides as follows. The endotoxin was injected intravenously in the marginal ear vein of a New Zealand albino rabbit weighing 1.5 to 2.0 kg; 100 μg of epinephrine (Epinephrine-HCl, 1:1000; Lederle Laboratories, Pearl River, N.Y.) in 0.1 ml were injected intradermally into the lateral shaved skin of the rabbit that had received endotoxin or saline (control) 30 min before. Skin tests were recorded after 18 to 24 hr as either positive or negative for intradermal hemorrhage (Buccino, Lingley, and Israel, 1962).

Chemical analysis. Carbohydrate was estimated by the anthrone method with galactose as standard (Shields and Burnett, 1960). Total lipid content of lipopolysaccharides was determined by methods previously described (Mergenhagen, Martin, and Schiffmann, 1963). Protein was determined by the method of Lowry et al. (1951).

Results and Discussion

The morphological and biochemical characteristics of all strains of bacteria utilized in this study conform to the description of either *F. polymorphum* (Omata and Braunberg, 1960) or *L. buccalis* (Gilmour et al., 1961).

An endotoxin was extracted by phenol-water treatment of cells from all strains of *F. polymorphum* and *L. buccalis*. Chemical analyses of a representative number of these endotoxins are shown in Table 1. All are low in protein content. The high lipid content of the preparations from both groups of organisms is of interest in view of recent experiments implicating the lipid as the component responsible for certain host responses to lipopolysaccharides (Westphal et al., 1958; Haskins et al., 1961; Mergenhagen et al., 1963).

The EST is a sensitive and highly reproducible bioassay for endotoxic activity. The alteration of dermal reactivity to epinephrine by intravenously injected *F. polymorphum* or *L. buccalis* endotoxin is shown in Table 2. The limiting intravenous dose of 1.0 μg for a positive EST demonstrates that these endotoxins have the same order of potency as those isolated by similar methods from Enterobacteriaceae (Buccino et al., 1962). By determining minimal lethal mouse doses of representative endotoxins as a second

TABLE 1. *Chemical composition of some Fusobacterium polymorphum and Leptotrichia buccalis lipopolysaccharides*

Source of lipopolysaccharide	Composition (%)		
	Saccharide	Lipid	Protein
F. polymorphum			
ATCC 10953	30.4	32.2	2.0
FEv4	26.4	37.2	6.8
FAL	58.8	37.3	3.6
LT5	22.4	32.3	5.2
SJ3	62.4	27.6	2.4
B6	54.4	19.7	2.8
SJ2	26.4	22.4	4.4
LT2	18.6	31.2	2.8
F1	48.8	26.2	3.6
L. buccalis			
C1100	37.2	42.8	1.6
C1170	24.4	30.4	2.4

TABLE 2. *Influence of endotoxins from Fusobacterium polymorphum and Leptotrichia buccalis on dermal reactivity of the rabbit to epinephrine*

Source of endotoxin	Dose (intravenously 30 min before epinephrine)				
	25 μg	10 μg	5 μg	1 μg	0.1 μg
F. polymorphum					
ATCC 10953	2/2*	2/2	1/3	2/3	0/3
B6	2/2	2/2	3/3	1/3	0/3
FAL	1/2	1/2	—	—	—
FRP	2/2	2/2	—	—	—
GS1	1/2	1/2	—	—	—
FEv4	1/2	1/2	—	—	—
B10	2/2	2/2	—	—	—
L. buccalis					
C1100	2/2	2/2	2/3	2/3	0/3
C1170	2/2	2/2	1/3	2/3	0/3
Saline	0/2	0/2	—	—	—

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

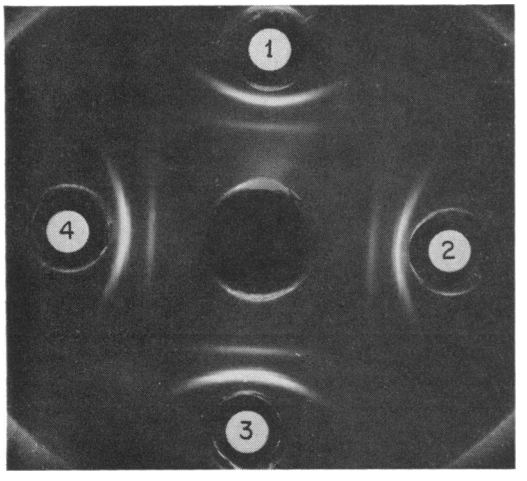

FIG. 1. *Agar gel diffusion pattern of Fusobacterium purified lipopolysaccharide. Undiluted serum (anti-B6) was in the center well. Different concentrations of lipopolysaccharide from strain B6 were in the numbered wells; 1 to 4 contained 2, 3, 4, and 5 mg per ml, respectively.*

measure of biological activity, we observed that the endotoxins from *L. buccalis* were more potent (strain C1170, LD_{50} = 260 μg; strain C1100, LD_{50} = 600 μg) than representative preparations from *F. polymorphum* (strain FBI, LD_{50} = 860 μg; strain FAL, LD_{50} = >1000 μg). These observations show that while such endotoxin preparations appear to be equally potent in one bioassay procedure (EST), another procedure (mouse lethality) may reveal differences in their potency.

Agar gel double-diffusion tests with several *Fusobacterium* endotoxins and antisera to the homologous organisms disclosed a rapidly diffusing component (precipitation band closer to serum well) and a second component which diffused more slowly (Fig. 1). This pattern of precipitation is similar to that obtained by Ribi et al. (1962) with endotoxin from *Salmonella enteriditis*. In like manner, the two precipitation bands displayed in the present study may be related to the haptenlike and residual endotoxin components described by those investigators. By centrifugation of a *F. polymorphum* endotoxin (strain B6) at 60,000 × *g* for 4 hr (Ribi et al., 1962), the two immunologically distinct components were readily separated as demonstrated by agar gel diffusion (Fig. 2); the sediment consisted of the slowly diffusing fraction, whereas

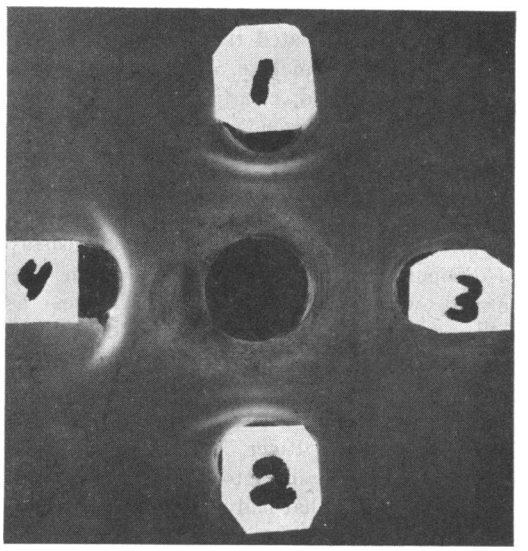

FIG. 2. *Agar gel diffusion plate of purified lipopolysaccharide (strain B6) and separated fractions by centrifugation at 60,000 × g for 4 hr with antiserum B6. Undiluted serum was in the center well. Wells 1 and 2 contained 1 mg and 2 mg per ml of purified endotoxin; 3 contained 2 mg per ml of supernatant fraction; 4 contained 2 mg per ml of deposit fraction.*

the supernatant contained the more rapidly diffusing component. Both fractions had haptenic activity. That is, both materials sensitized sheep erythrocytes, so that they were agglutinated by strain B6 antiserum. Both fractions provoked extensive dermal necrosis in rabbits (EST) in concentrations of 5 μg. The relationship of each of these immunologically distinct components to the parent material extracted with phenol-water requires further investigation.

Hemagglutination-inhibition tests carried out with purified lipopolysaccharides revealed that there are numerous serologically specific lipopolysaccharides in both groups studied. From the condensed data in Table 3, several serological types comprised the *F. polymorphum* and *L. buccalis* strains studied. These results, demonstrating the high degree of serological specificity of the lipopolysaccharides, are analogous to those reported for the genus *Veillonella* by Mergenhagen and Varah (1963). An interesting point is that none of the *F. polymorphum* lipopolysaccharides used in this study shared a serological relationship to the lipopolysaccharide extracted from the *F. polymorphum* type-strain (ATCC 10953), although all of the strains were homogeneous biochemically.

The presence of a common antigen in the supernatants of heated cells from three *F. polymorphum* strains was studied by means of the hemagglutination reaction (Table 4). Sheep erythrocytes treated with "crude antigen" from three strains of *F. polymorphum* (ATCC 10953, B6, and B10) were agglutinated by heterologous as well as homologous antisera prepared against several strains of *F. polymorphum*, with the exception of antiserum to strain B6 which failed to agglutinate erythrocytes sensitized with strain 10953 "crude antigen." Antisera prepared against two strains of *L. buccalis* (C1100 and C1170) failed to agglutinate cells sensitized with *F. polymorphum* "crude antigen." Cell-free extracts prepared from the above strains in a French pressure cell contained cross-reacting antigens qualitatively similar to those contained in the "crude antigen" prepared by heat treatment of the cells.

With "crude antigen" from four strains of *L. buccalis*, including two freshly isolated human oral strains, only antisera prepared against the *L. buccalis* strains agglutinated erythrocytes sensitized with such antigen mixtures, and antisera prepared against *F. polymorphum* failed to react (Table 4).

TABLE 3. *Agglutination by homologous antiserum of sheep erythrocytes sensitized with purified lipopolysaccharides from Fusobacterium polymorphum and Leptotrichia buccalis and its inhibition by homologous and heterologous lipopolysaccharides**

Source of lipopolysaccharide	Lipopolysaccharides exhibiting significant inhibition	
	F. polymorphum	*L. buccalis*
F. polymorphum		
B10	B10, B6	
B6	B10, B6	
FAL	FAL	
SJ3	SJ3, SJ2, FRP, FRP(1)	
SJ2	SJ3, SJ2, FRP, FRP(1)	
GS1	GS1	
FRP	SJ3, SJ2, FRP, FRP(1)	
FRP(1)	SJ3, FRP, FRP(1)	
ATCC 10953	ATCC 10953	
FEv1	FEv1	
FEv4	FEv4, F1, F2	
F3	F3, FJ1	
FJ1	FJ1	
LT1	LT1, LT2, LT3	
LT2	LT2, LT3, LT6	
LT3	LT2, LT3, LT6, LT5	
FB1	FB1	
L. buccalis		
C1100		C1100
C1170		C1170, C1013b
C1327		C1327
C1013b		C1100, C1013b

* Each of the 27 lipopolysaccharides was used in hemagglutination-inhibition tests.

The gel diffusion pattern with "crude antigen" from *F. polymorphum* (ATCC 10953) disclosed three or sometimes four bands of precipitation (Fig. 3). Two of them are related to the fast-moving and slow-moving components described with the purified lipopolysaccharide. Antiserum absorbed with homologous lipopolysaccharide precipitated only the center band in agar gel diffusion tests. That is, the slowest- and fastest-moving components disappeared after absorption

TABLE 4. *Agglutination of sheep erythrocytes sensitized with "crude antigen" from Fusobacterium polymorphum and Leptotrichia buccalis by homologous and heterologous antisera**

"Crude antigen"† on red blood cells	Antisera											
	F. polymorphum								*L. buccalis*			
	B10	FAL	B6	SJ3	GS1	FRP	ATCC 10953	FEv4	C1100	C1170	C1327	C1013b
F. polymorphum												
ATCC 10953	6400	1600	0	1600	12,800	3200	51,200	12,800	0	0	—	—
B6	25,600	800	800	1600	3200	800	6400	1600	0	0	—	—
B10	25,600	1600	3200	3200	6400	1600	6400	3200	0	0	—	—
L. buccalis												
C1100	100‡	100	100	100	100	100	50	100	25,600	25,600	25,600	25,600
C1327	100	100	100	100	100	100	0	50	25,600	12,800	25,600	25,600
CA3	200	100	200	100	200	100	50	200	25,600	25,600	25,600	25,600
CA5	100	100	100	100	100	100	50	100	25,600	25,600	25,600	25,600

* Results expressed as hemagglutination titer (reciprocal).

† Soluble portion of cells in 0.5 M phosphate buffer after heating cells at 100 C for 1 hr.

‡ Some normal rabbit sera at dilutions of 1:200 agglutinated cells sensitized with "crude antigen" from *L. buccalis*.

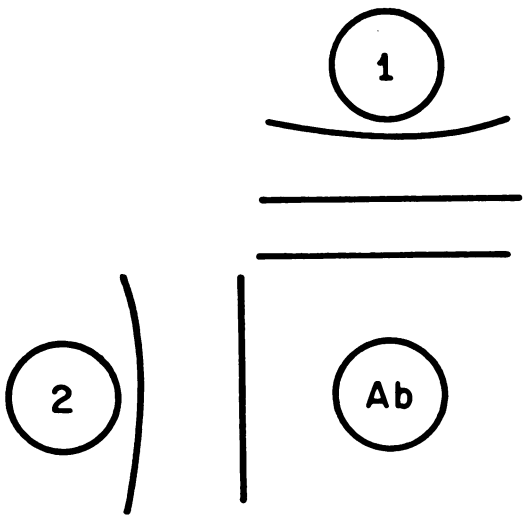

FIG. 3. *Agar gel diffusion plate of "crude antigen" and lipopolysaccharide from Fusobacterium polymorphum ATCC 10953. Ab, undiluted serum prepared against F. polymorphum; 1, 2 mg per ml of homologous "crude antigen"; 2, 2 mg per ml of homologous endotoxin.*

of the serum with purified lipopolysaccharide. One can conclude from these experiments that the remaining precipitation band after absorption of antiserum with lipopolysaccharide contains the antigen or antigens responsible for group reactivity.

Phenol treatment of *F. polymorphum* ATCC 10953 "crude antigen," or prior treatment with trypsin followed by dialysis, produced a product that displayed serological specificity analogous to that found with the purified lipopolysaccharide (Table 5). These experiments provide strong evidence that the group reactivity of the *F. polymorphum* "crude antigen" is dependent upon the protein nature of the antigen or antigens. Before treatment with phenol, the "crude antigen" contained approximately 50% protein; after treatment it contained less than 5%. Deproteinization, therefore, leaves a type-specific reactivity presumably due to the lipopolysaccharide in the "crude antigen." The hemagglutination reaction using unaltered erythrocytes sensitized with "crude antigen" from *F. polymorphum* probably requires a polysaccharide to modify the surface of the erythrocyte (Neter, 1956). It is quite possible that the group-reactive protein is attached to erythrocytes by means of a polysaccharide-protein complex present in the "crude antigen."

In contrast to findings with *F. polymorphum*, "crude antigen" extracted from *L. buccalis* did not lose group-reactivity with heterologous antisera after phenol or trypsin treatment (Table 6).

TABLE 5. *Agglutination of sheep erythrocytes sensitized with antigens from Fusobacterium polymorphum ATCC 10953 by various Fusobacterium polymorphum and Leptotrichia buccalis antisera**

Antigens from *F. polymorphum* ATCC 10953 on red blood cells	Antisera																
	F. polymorphum															*L. buccalis*	
	B10	FAL	B6	SJ3	GS1	FRP	ATCC 10953	FEv4	FRP(1)	FEv1	FJ1	LT2	LT3	FB1	LT1	SJ2	C1100	C1170
Purified lipopolysaccharide	0	0	0	100	0	0	25,600	200	50	0	0	0	0	0	0	0	50	0
"Crude antigen"	6400	1600	0	1600	12,800	3200	51,200	12,800	3200	200	800	100	6400	400	400	12,800	0	0
"Crude antigen" after phenol treatment	0	0	0	100	0	0	25,600	400	0	0	0	0	0	0	0	1600	200	0
"Crude antigen" after trypsin treatment	0	0	0	100	0	0	25,600	400	0	0	0	0	0	0	0	800	100	0

* Results expressed as hemagglutination titer (reciprocal).

TABLE 6. *Agglutination of sheep erythrocytes sensitized with antigens from Leptotrichia buccalis C1100 by homologous and heterologous antisera**

Antigens from *L. buccalis* C1100 on red blood cells	Antisera			
	C1100	C1170	C1327	C1013b
Purified lipopolysaccharide	6400	0	0	0
"Crude antigen"†	25,600	12,800	25,600	25,600
"Crude antigen" after phenol treatment	25,600	12,800	25,600	25,600
"Crude antigen" after trypsin treatment	25,600	25,600	6400	25,600

* Results expressed as hemagglutination titer (reciprocal).

† Soluble portion of cells in 0.5 M phosphate buffer after heating cells at 100 C for 1 hr.

These results suggest that the determinant group antigen in *L. buccalis* is polysaccharide in nature.

The results reported here provide immunochemical evidence that *L. buccalis* (*F. fusiforme*) is distinct from *F. polymorphum* strains. These findings strongly support the proposed separation of the two genera, formerly based upon morphological and biochemical activities (Bøe, 1941; Jackins and Barker, 1951; Omata and Braunberg, 1960). On the other hand, the fact that *L. buccalis* contains toxic lipopolysaccharides with high lipid contents raises some doubt as to the validity of including this genus in the family Lactobacillaceae (Hamilton and Zahler, 1957; Gilmour et al., 1961; Rosebury, 1962), since cell-wall components with high lipid contents are thought to be characteristic of gram-negative bacteria (Salton, 1960).

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