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Actinobacillus actinomycetemcomitans Serotype b Lipopolysaccharide Mediates Coaggregation with *Fusobacterium nucleatum*

Graciela Rosen, Ira Nisimov, Monica Helcer, and Michael N. Sela*

Department of Oral Biology, Hebrew University-Hadassah Faculty of Dental Medicine, Jerusalem, Israel

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Purified *Actinobacillus actinomycetemcomitans* serotype b lipopolysaccharide (LPS) was found to be able to bind *Fusobacterium nucleatum* cells and to inhibit binding of *F. nucleatum* to *A. actinomycetemcomitans* serotype b. Sugar binding studies showed that the requirements for binding of *A. actinomycetemcomitans* serotype b LPS to the *F. nucleatum* lectin are the presence of a metal divalent ion, an axial free hydroxyl group at position 4, and free equatorial hydroxyl groups at positions 3 and 6 of D-galactose, indicating that the β-*N*-acetyl-D-galactosamine in the serotype b LPS trisaccharide repeating unit is the monosaccharide residue recognized by the *F. nucleatum* lectin. These data strongly suggest that *A. actinomycetemcomitans* serotype b LPS is one of the receptors responsible for the lactose-inhibitable coaggregation of *A. actinomycetemcomitans* to fusobacteria.

Lactose-inhibitable coaggregation is a common interaction among oral bacteria, including periodontal microorganisms such as *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum*. Binding of these two bacteria is mediated by a galactoside moiety on the *A. actinomycetemcomitans* surface and a lectin on *F. nucleatum.* Protease treatment or heating the *F. nucleatum* at 85°C completely prevents coaggregation with *A. actinomycetemcomitans.* On the other hand, when these treatments were applied to the *A. actinomycetemcomitans* partner, coaggregation was not affected (9).

A. actinomycetemcomitans is a nonmotile, gram-negative capnophilic, fermentative coccobacillus that has been implicated in the etiology and pathogenesis of juvenile (30) and adult (24) periodontitis as well as systemic infections (20). *A. actinomycetemcomitans* strains isolated from the human oral cavity are divided into six serotypes, a to f (3, 7, 19, 31). The serotype-specific antigens are major targets of the humoral response in periodontitis patients colonized by these species (1, 22). These antigens are located in the O-polysaccharide (O-PS) region of the lipopolysaccharide (LPS) (16, 18, 27). The chemical structures of the *A. actinomycetemcomitans* serotype a to f antigenic O-PSs were determined (7, 17, 18), and the DNA sequences of the genes involved in their synthesis have been described previously (7, 14, 15, 25, 28, 29). The structural differences between these antigens are the basis for the absence of cross-reactivity among the different *A. actinomycetemcomitans* serotypes (1, 22), with the exception of serotypes b and f, which show serological cross-reactivity, probably due to a common β-*N*-acetyl-galactosamine epitope (7).

Of these strains, serotype b is most frequently isolated from subjects with localized juvenile periodontitis (30, 31), who exhibit elevated serum antibody levels to serotype b-specific antigen (1, 22). The serotype b O-PS region of the LPS (18) consists of a polymer of repeating trisaccharide units with the structure →3)α-D-Fuc*p*-(1→2)-3-O-(β-D-Gal*p*NAc)-α L-Rha*p*(1→.

* Corresponding author. Mailing address: Department of Oral Biology, Faculty of Dental Medicine, the Hebrew University, P.O. Box 12272, Jerusalem 91120, Israel. Phone: 972-2-6758597. Fax: 972-2-6438335. E-mail: msela@cc.huji.ac.il.

F. nucleatum strains are the most numerous gram-negative bacteria isolated from healthy periodontal sites and are the most common predominant pathogen in subsequent periodontal destruction (4, 13). *F. nucleatum* strains were shown to be able to coaggregate all species of oral bacteria tested (9, 10) and thus play an important part in the development of dental plaque.

Two different galactose-binding adhesins of *F. nucleatum* were proposed to be responsible for the lactose-inhibitable coaggregation with *Porphyromonas gingivalis* (9) and to its attachment to mammalian cells (26): a major 42-kDa membrane protein (8) and a surface 30-kDa polypeptide extracted from the surface of the bacteria (21). While these preliminary studies were focused on the characterization of the *F. nucleatum* adhesins, there have been no reports on the identification and characterization of the complementary receptors on the gram-negative anaerobic partners. The aim of the present study was to examine the role of LPS from *A. actinomycetemcomitans* serotype b as a possible receptor for the lactose-inhibitable coaggregation with *F. nucleatum*. The minimal carbohydrate structural requirements for recognition by the *F. nucleatum* galactose-binding lectin are also reported.

(Work by I.N. was performed as part of the M.Sc. degree at Hebrew University, Jerusalem, Israel.)

A. actinomycetemcomitans strains Y4, JP2 (serotype b), and ATCC 29523 (serotype a) were grown as previously described (23) at 37°C in 5% CO_2. *F. nucleatum* PK 1594 was grown in Wilkins-Chalgren anaerobe broth (Oxoid) at 37°C under anaerobic conditions. For the coaggregation or binding assays, bacterial cells were harvested, washed with coaggregation buffer (CB; 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3), and stored at 4°C until used.

LPS was prepared as previously described (18). After ultracentrifugation, the LPS was further purified by gel filtration on a Sephacryl S-400 HR (900 by 16 mm; Pharmacia Fine Chemicals in an AKTA explorer system; Amersham Biosciences) at room temperature with disaggregation buffer (0.05 M Tris-HCl [pH 9.0], 0.001 M EDTA, 0.3% deoxycholate) as eluent. Fractions containing LPS were identified by silver staining and

precipitated by addition of 0.15 M NaCl and 4 volumes of 95% ethanol. The precipitates were isolated by centrifugation at 12,000 × *g* for 20 min at 4°C, pooled, dissolved in water, dialyzed against water, and lyophilized. The O-PS was prepared and purified as described by Perry et al. (18). The high-molecular-weight fraction obtained from the Sephadex G-50 column chromatography contained the O-PS (K_{av}, 0.04).

Coaggregation was routinely assayed by the visual coaggregation assay as described by Kolenbrander et al. (9). Coaggregation scores from 0 to 4+ were monitored visually according to the scale described by Cisar et al. (2).

Coaggregate formation by accretion onto a partner cell-coated microtiter well surface was adapted from the assay described by Jenkinson et al. (6). Unlabeled *A. actinomycetemcomitans* cells, adjusted to a density of 10^8 cells per ml in CB, and 50-μl samples were applied to the wells of 96-well microtiter plates (Maxisorp, Nunc, Denmark). The plates were centrifuged at 800 × *g* at 20°C for 5 min and further incubated at 4°C for 16 h. The plates were blocked for 2 h at room temperature by adding 200 μl of 0.4% Tween 20 in CB.

Radioactively labeled samples of 50 μl of [^{3}H]*N*-acetyl-glucosamine-labeled *F. nucleatum* cells (1.7×10^7 cells; specific radioactivity about 10^3 cells per cpm) were added to the wells, and the plates were incubated for 2 h on a rotary shaker. The wells were washed four times with 0.05% Tween 20 in CB. Accreted cells were removed from the plastic surface by adding 100 μl of a solution containing 1% sodium dodecyl sulfate and 0.4 M NaOH for 2 h and transferring the liquid contents for determination of radioactivity. The assays were performed in quadruplicate, and wells without the unlabeled partner were used as control wells.

Binding of ^{3}H-labeled *F. nucleatum* cells to either *A. actinomycetemcomitans* LPS or O-PS was tested by the same assay, but the plates were coated with either 100 μl of LPS or O-PS (100 μg/ml of CB). When the inhibitory effect of sugars, EDTA, or LPS was tested, the ^{3}H-labeled *F. nucleatum* cells were preincubated for 30 min at room temperature at the indicated concentrations before being added to the plates. All sugars were obtained from Sigma and are of the D-configuration and in pyranose form, unless otherwise indicated.

The percentage of inhibition was calculated as [(binding in the absence of inhibitor − binding in the presence of inhibitor)/binding in the absence of inhibitor] × 100.

For each of the measures (before and after inhibition), the mean, standard deviation, and coefficient of variation were calculated. Statistical analysis consisted of a two-tailed nonpaired *t* test for comparing the mean inhibition with *A. actinomycetemcomitans* ATCC 29523 LPS versus that with Y4 or JP2 LPS.

A. actinomycetemcomitans strains Y4 and JP2 (serotype b) coaggregated with *F. nucleatum* PK 1594, while *A. actinomycetemcomitans* ATCC 29523 (serotype a) showed no visible coaggregation (Table 1). The coaggregation between the two serotype b *A. actinomycetemcomitans* strains and *F. nucleatum* was completely inhibited by 10 mM galactose (Gal), while 20 mM glucose (Glc) was without effect. EDTA (2 mM) also completely inhibited coaggregation.

The purified LPSs from the two serotype b *A. actinomycetemcomitans* strains (Y4 and JP2) and the serotype a strain (ATCC 29523) as well as O-PS from strain JP2 were tested for their capacity to bind to *F. nucleatum*. LPSs from *A. actinomycetemcomitans* serotype b strains Y4 and JP2 were found to bind to *F. nucleatum* cells as compared to controls (without LPS) and to LPS from *A. actinomycetemcomitans* serotype a, which did not bind to *F. nucleatum* (Fig. 1). Furthermore, O-PS from *A. actinomycetemcomitans* JP2 bound *F. nucleatum* to the same extent as LPS from *A. actinomycetemcomitans* JP2 (Fig. 1). Binding of *F. nucleatum* to LPS from serotype b strains Y4 and JP2 was completely inhibited by 10 mM Gal or 2 mM EDTA (not shown for LPS from strain JP2), while no inhibition by 20 mM Glc could be observed (Fig. 1).

TABLE 1. Coaggregation of *F. nucleatum* PK 1594 and different *A. actinomycetemcomitans* strains in the presence of monosaccharides or EDTA

A. actinomycetemcomitans strain	Inhibitor addition[a]	Coaggregation score[b]
Y4	None	3
	Gal or GalNAc (10 mM)	0
	Glc (20 mM)	3
JP2	None	2
	Gal or GalNAc (10 mM)	0
	Glc (20 mM)	2
ATCC 29523	None	0
Y4 or JP2	EDTA (2 mM)	0

[a] Final inhibitor concentration.
[b] The coaggregation score was determined by the visual coaggregation assay (9).

The LPSs from *A. actinomycetemcomitans* serotype b strains Y4 and JP2 were tested for their capacity to interfere with the binding of *F. nucleatum* to *A. actinomycetemcomitans* cells. *A. actinomycetemcomitans* serotype a LPS was used as a control. As shown in Fig. 2, Y4 LPS inhibited binding of *F. nucleatum* to Y4 *A. actinomycetemcomitans* cells in a dose-dependent manner. Inhibition by serotype b LPS Y4 was significantly greater than the inhibition observed with serotype a LPS (75% inhibition for the Y4 LPS versus 25% inhibition for the ATCC 29523 LPS at 100 μg/ml). A similar dose-dependent inhibition was observed when JP2 LPS was used to inhibit binding of *F. nucleatum* to *A. actinomycetemcomitans* JP2 cells (Fig. 3): 70% inhibition for JP2 LPS versus 5% inhibition for the serotype a LPS at 100 μg/ml.

To study the interaction between the *F. nucleatum* lectin and the serotype b *A. actinomycetemcomitans* LPS receptor, the effect of a range of concentrations of different saccharides on binding of *F. nucleatum* to Y4 LPS was tested. For each compound examined, inhibition curves were constructed. Based on these curves, we estimated the concentration of each saccharide that inhibited binding of *F. nucleatum* to Y4 LPS by 50% (I_{50}). Table 2 shows the I_{50}s of the different carbohydrates. The best inhibitors of the *F. nucleatum* lectin were Gal, lactose, and related compounds with a free and axial hydroxyl group at position 4 and equatorial free hydroxyl groups at positions 3 and 6: *N*-acetyl-galactosamine (GalNAc), 2-deoxy-Gal, methyl-α-galactoside (α-MeGal), raffinose, and mellibiose. Glc and mannose (Man) at 100 mM and L-rhamnose (L-Rha) at 50 mM with an equatorial hydroxyl at position 4 could not inhibit bacterial binding. Furthermore, cellobiose was at least 60 times

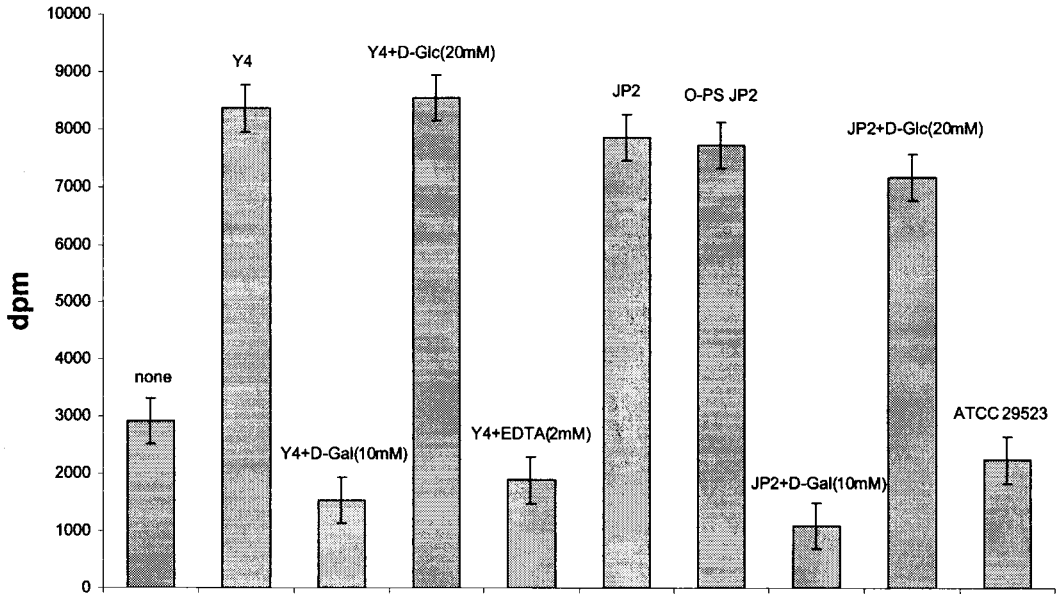

FIG. 1. Binding of ^{3}H-labeled *F. nucleatum* cells to LPS of *A. actinomycetemcomitans* strains Y4, JP2, and ATCC 29523 and to O-PS from strain JP2 in the absence or presence of monosaccharides and EDTA. Binding is expressed as radioactivity accreted to the wells.

less active than lactose. Taking into account that cellobiose is identical to lactose, except for the orientation of the 4-hydroxyl group in the nonreducing sugar (Gal in lactose and Glc in cellobiose), it is reasonable that the lectin interacts with the 4-hydroxyl group of Gal. This is also indicated by the loss of inhibitory activity of the galactose derivative substituted at position 4, Gal-4 sulfate. D-Gulose, the 3-epimer of Gal, had also no inhibitory effect, indicating that the interaction probably involves the equatorial orientation of the 3-hydroxyl group of Gal. In contrast, Gal derivatives with substituents at position 2 were substantially as inhibitory as Gal: GalNAc and 2-deoxy-Gal showed I_{50}s comparable to those of Gal. Position 6 of Gal also appears to be important for binding, since fucose (Fuc; 6-deoxy-galactose) was 22 times less active than Gal, and the derivative with a negatively charged group at this position (Gal-6 sulfate) was virtually inactive. Structural analysis of LPS from the serotype a *A. actinomycetemcomitans* strain (ATCC 29523) indicated that its O-PS contains 6-deoxy-D-talose (6dTal*p*) and *O*-acetyl (2:1) and is a polymer of disaccharide repeating units with the structure:→3)-α-D-6dTal*p*(1→2)-α-D-

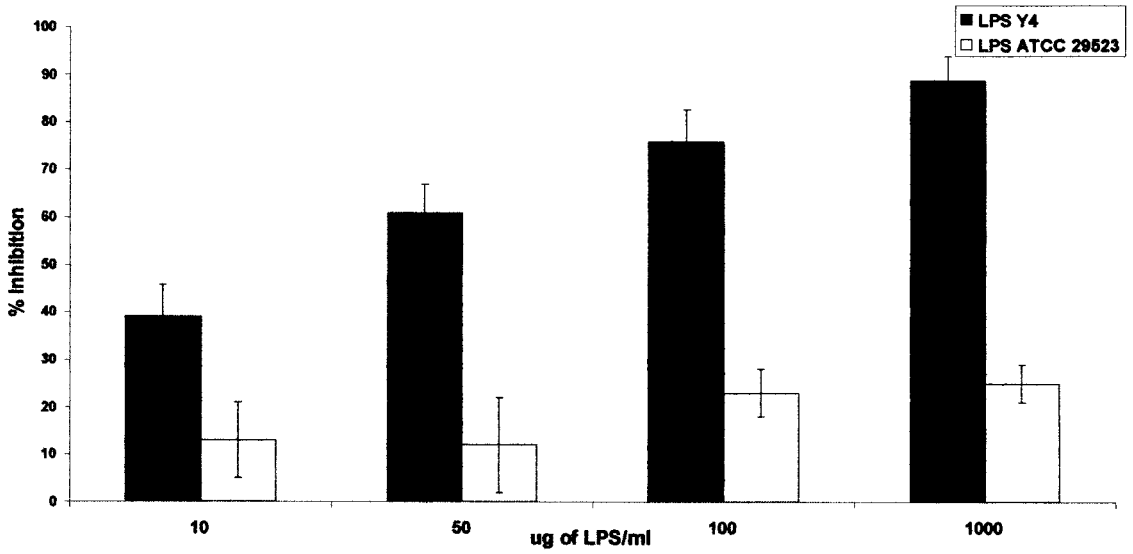

FIG. 2. Inhibition of binding of ^{3}H-labeled *F. nucleatum* cells to Y4 *A. actinomycetemcomitans* cells at the indicated Y4 LPS concentrations. $P < 0.01$ for inhibition by *A. actinomycetemcomitans* Y4 LPS versus ATCC 29523 LPS at 10, 50, and 100 μg/ml; $P < 0.001$ for inhibition by LPS at 1,000 μg/ml.

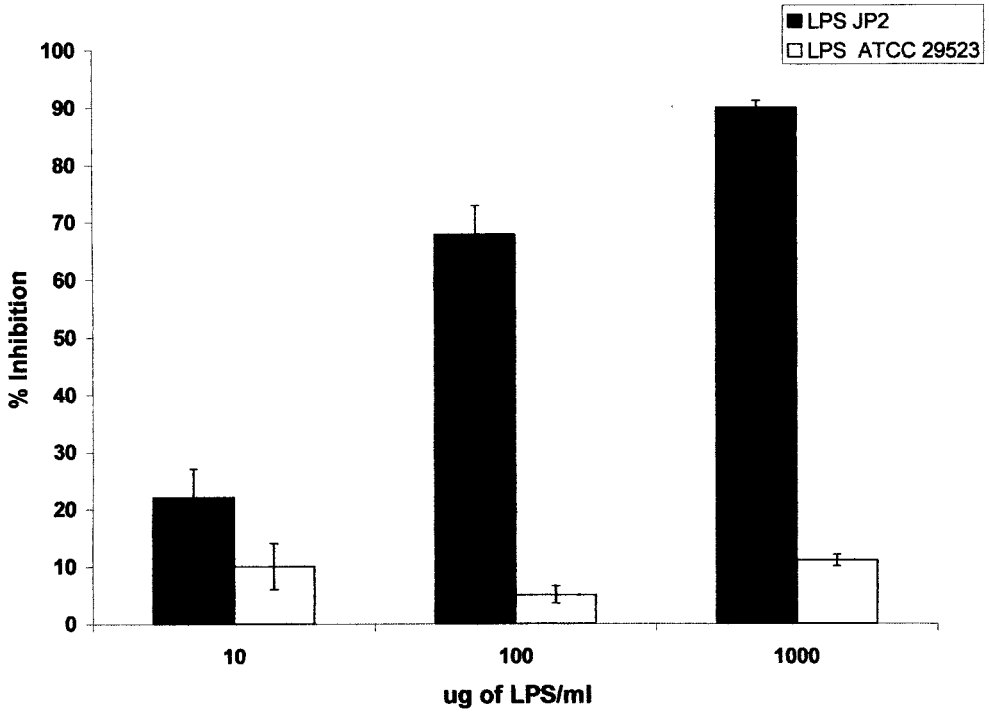

FIG. 3. Inhibition of binding of ^{3}H-labeled *F. nucleatum* cells to JP2 *A. actinomycetemcomitans* cells at the indicated JP2 LPS concentrations. $P < 0.05$ for inhibition by *A. actinomycetemcomitans* JP2 LPS versus ATCC 29523 LPS at 10 μg/ml; $P < 0.001$ for inhibition by LPS at 100 and 1,000 μg/ml.

6dTal*p*-(1→ (17). *F. nucleatum* cells neither coaggregate with *A. actinomycetemcomitans* strain ATCC 29523 nor bind to its LPS. Although talose is the 2-epimer of Gal, a position that is not necessary for binding, the substitution of the C-6 hydroxyl group probably renders the *A. actinomycetemcomitans* serotype a LPS incapable of binding to *F. nucleatum* under our experimental conditions. α-MeGal, raffinose, mellibiose, and lactose also had the same I_{50}s as Gal, indicating that substitution at the anomeric carbon atom (α or β) does not influence the binding activity. The comparable I_{50}s of lactose and Gal also indicate that the hydroxyl groups of Glc do not participate in the binding, as in the case of mammalian galectins (5).

TABLE 2. Inhibition of binding of *F. nucleatum* to Y4 LPS by a series of saccharides

Inhibitor	Formula	I_{50} (mM)[a]
Gal		0.78
GalNAC		0.78
2-Deoxy-Gal		0.85
Gal-4-sulphate		NI (20)
Gal-6-sulphate		NI (50)
Gulose		NI (50)
Fuc		17
L-Rha		NI (50)
Glc		NI (100)
Man		NI (100)
α-MeGal		0.78
Lactose	Gal β1-4Glc	0.78
Raffinose	Gal α1-6Glc β1-2Fruc$_f$	0.78
Mellibiose	Gal α1-6Glc	0.78
Cellobiose	Glc β1-4Glc	NI (50)

[a] I_{50} is the inhibitor concentration that caused 50% reduction in binding. NI, not inhibitory at the millimolar concentration shown in parentheses.

The present results demonstrate that serotype b *A. actinomycetemcomitans* LPS acts as a receptor for coaggregation with *F. nucleatum*. This conclusion is supported by the ability of serotype b LPS to bind to cells of *F. nucleatum* and its inhibitory effect on the binding of *F. nucleatum* to *A. actinomycetemcomitans* cells.

Different serotypes of *A. actinomycetemcomitans* differ in their ability to coaggregate with *F. nucleatum* and in the chemical structure of the O-PS moieties of their LPSs (17, 18). The lipid A and core polysaccharide structures of the LPS were found to be identical among the different serotypes (12, 18). The serotype b *A. actinomycetemcomitans* LPS is capable of binding *F. nucleatum*, probably through its galactose-binding lectin. Our inhibition studies suggest that the most important characteristics of the binding site of this lectin are as follows. (i) It is dependent on a divalent metal ion for its carbohydrate binding activity, since EDTA completely inhibited binding, thus resembling the ion requirements of the C-type animal lectins (11). (ii) A free axial hydroxyl group at position 4 and free equatorial hydroxyl groups at positions 3 and 6 of Gal are necessary for binding.

To our knowledge, this is the first report identifying polysaccharide receptors for coaggregation on the surface of gram-negative late colonizers of the dental plaque.

In summary, the results of the present study indicate that LPS from *A. actinomycetemcomitans* cells plays a role in their attachment to other microorganisms in dental plaque, thus

creating a reservoir of bacteria that are involved in the pathogenesis of periodontal as well as systemic diseases. Furthermore, knowledge of the structural requirements of the galactose-binding lectin may lead to the development of derived saccharides that may be used as inhibitors of coaggregation and therein point to a mechanism for inhibiting subgingival plaque formation.

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