Coaggregation of Porphyromonas gingivalis and Fusobacterium nucleatum PK 1594 is mediated by capsular polysaccharide and lipopolysaccharide

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Abstract
Previous reports have shown that coaggregation between Porphyromonas gingivalis and Fusobacterium nucleatum, two important periodontopathogens, is mediated by a galactoside on the surface of P. gingivalis and a lectin on F. nucleatum. In the present study, purified capsular polysaccharide (CPS) and lipopolysaccharide (LPS) of P. gingivalis PK 1924 (serotype K5) were found to be able to bind to F. nucleatum cells and to inhibit binding of F. nucleatum to P. gingivalis serotype K5. Sugar binding studies showed that the requirements for binding of P. gingivalis serotype K5 CPS and 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 position 3 and 6 of D-galactose. These data suggest that P. gingivalis serotype K5- CPS and LPS act as receptors mediating coaggregation between P. gingivalis and fusobacteria.

Introduction
Porphyromonas gingivalis are Gram-negative oral anaerobic bacteria that have been implicated as important etiological agents in the development of periodontal diseases (Haffajee & Socransky, 1994). The pathogenic role of these bacteria in the disease process depends on their ability to bind to host cells as well as to other bacteria in the subgingival biofilm, their ecological site in the oral cavity. One of the main mechanisms of bacterial binding in the subgingival plaque occurs through coaggregation. Fusobacterium nucleatum, which are also Gram-negative anaerobes associated with periodontal diseases (Moore et al., 1982, 1985) and failing implants (Lisgarten & Lai, 1999) were shown to coaggregate with all species of oral bacteria tested (Kolenbrander & Anderson, 1989; Kolenbrander et al., 1989; Kolenbrander & London, 1993) including the late colonizers P. gingivalis and Actinobacillus actinomycetemcomitans, thus playing a central role in the development of the dental plaque.

Coaggregation between P. gingivalis PK 1924 and F. nucleatum PK 1594 is mediated by a galactoside moiety on the P. gingivalis surface and a lectin on the F. nucleatum, and inhibited by lactose, galactose and related monosaccharides (Kolenbrander & Anderson, 1989; Kinder & Holt, 1993; Shanitzki et al., 1997). Polysaccharides (including lipopolysaccharides (LPS) and lipo-oligosaccharides) of various microorganisms were shown to be involved in their adherence to host cells as well as other bacteria (McIntire et al., 1988; Jaques, 1996; Rosen et al., 2003).

We have recently reported that the lactose inhibitable coaggregation between F. nucleatum and A. actinomycetemcomitans serotype b (strains Y4 and JP2) is mediated by the A. actinomycetemcomitans serotype b LPS (Rosen et al., 2003). We have also showed that the requirements for binding of serotype b A. actinomycetemcomitans LPS to the F. nucleatum PK 1594 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 (D-Gal). This may indicate that the β-N-acetyl-D-galactosamine (GalNAc)in the serotype b LPS trisaccharide repeating unit is the monosaccharide residue recognized by the F. nucleatum PK 1594 lectin (Perry et al., 1996; Rosen et al., 2003).

Three different polysaccharides of P. gingivalis ATCC 53978 (formerly P. gingivalis W50) were reported: an extra-cellular polysaccharide, and two surface cellular associated polysaccharides: an acidic capsular polysaccharide (CPS) and the LPS (Farquharson et al., 2000). The classification of
Porphyromonas gingivalis strains into six different K serotypes (K1 to K6) is based on their CPS and K-, to strains devoid of CPS (Laine et al., 1997; Sims et al., 2001).

In the light of the different abilities of the Porphyromonas gingivalis K serotypes to coaggregate with fusobacteria and the differences in the chemical structure of their CPS and the O-PS moiety of their LPS, the aim of the present study was to examine the role of Porphyromonas gingivalis CPS and LPS, as possible receptors for coaggregation with Fusobacterium nucleatum.

Materials and methods

Bacterial strains and culture conditions

Porphyromonas gingivalis PK 1924, ATCC 53978, ATCC 33277 and W381, and Fusobacterium nucleatum PK 1594 were grown in Wilkins–Chalgren anaerobe broth (Oxoid Ltd, Basingstoke, UK) at 37 °C under anaerobic conditions (85% N2–10% H2–5% CO2). Actinobacillus actinomycetemcomitans strains Y4, (serotype b) and ATCC 29523 (serotype a) were grown as previously described (Slots, 1982). For the coaggregation or binding assays, cells were harvested, washed with coaggregation buffer (CB), 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2 and 0.02% NaN3) and stored at 4 °C until used.

Extraction and purification of CPS and LPS

Extraction and purification of Porphyromonas gingivalis CPSs and LPSs were performed as previously described (Schifferle et al., 1989; Choi et al., 1998). LPS from Actinobacillus actinomycetemcomitans strains Y4, (serotype b) and ATCC 29523 (serotype a) were extracted and purified as described before (Rosen et al., 2003).

Capsular polysaccharides from strains W50, HG184, A7A1-28, 49417, HG1690 and HG1691 represent capsular serotype K1 to K6 (Laine et al., 1997), respectively. The capsular serotype of Porphyromonas gingivalis strain PK 1924 was determined by double immunodiffusion (Outcherlony) using 1% Agar Noble (Difco, Detroit, MI) in Veronal buffer, pH 8.0.

ELISA

Capsular polysaccharide or LPS (100 μg mL−1) were incubated overnight at 4 °C in Maxisorp Nunc-Immunoplates (Nunc-maxisorp, Roskilde, Denmark). After blocking with 5% albumin (2 h at 37 °C), glycine-peroxidase lectin (Sigma, St Louis, MO) was added for 1 h at RT, washed, and detected with peroxidase substrate.

Coaggregation assays

(1) Visual coaggregation was routinely assayed as described by Kolenbrander et al. (1989). Coaggregation scores from 0 to 4+ were then followed visually, using the scale described by Cisar et al. (1979). (2) [3H]-labeled coaggregates: un-labeled Porphyromonas gingivalis cells were adjusted to a density of 1 x 10^8 cells mL⁻¹ in CB and 50 μL samples were applied to the wells of microtiter plates. Binding of [3H]-N-acetylglucosamine–F. nucleatum (1.7 x 10^7 cells; specific radioactivity about 10^5 cells per c.p.m.) to the Porphyromonas gingivalis cells was tested as described before (Rosen et al., 2003). The assays were performed in quadruplicates and control wells were without the unlabeled partner. Binding of [3H]-labeled F. nucleatum cells to either Porphyromonas gingivalis CPS or LPS (100 μg mL⁻¹ CB) was tested by the same assay. When the inhibitory effect of monosaccharides, EDTA, CPS or LPS was tested the [3H]-labeled F. nucleatum cells were preincubated for 30 min at RT before addition to the plates.

Statistical analysis

Statistical significance between groups was evaluated by ANOVA followed by the Scheffe multiple-comparison test. Differences between groups were considered significant at the level of P < 0.05.

Results

Fusobacterium nucleatum PK1594 coaggregated strongly with Porphyromonas gingivalis PK 1924, with a maximal coaggregation score of 4+. Coaggregation between these two bacteria was inhibited by 10 mM β-Gal or EDTA while 20 mM d-glucose (d-Glc) had no effect (Table 1) and was equally effective in the presence of either Ca2⁺ or Mg2⁺ ions. Double diffusion studies by the method of Outcherlony using polyclonal rabbit antiserum against each of the six Porphyromonas gingivalis K serotypes showed that Porphyromonas gingivalis PK 1924 belongs to the K5 serotype (data not shown). Porphyromonas gingivalis ATCC 53978 (capsular serotype K1) did not coaggregate with F. nucleatum PK 1594. Porphyromonas gingivalis W381 and ATCC 33277 (two nonencapsulated strains, K-) were found to form extensive autoaggregates when examined visually, either when the coaggregation assay was performed in PBS or in CB (Table 1). Phase microscope examination after the addition of both partners showed aggregates of Porphyromonas gingivalis as well as eventual adherence of F. nucleatum to the aggregates (not shown).

Coaggregation between F. nucleatum PK 1594 and different K serotypes of Porphyromonas gingivalis was further evaluated by accretion of radioactively labeled bacteria onto cell-coated microtiter plates (Table 1). By this methodology binding of [3H]-F. nucleatum showed a coaggregation pattern similar to the visual coaggregation assay (Table 1), and the effect of autoaggregation was avoided. Coaggregation of F. nucleatum with the two noncapsulated Porphyromonas gingivalis strains W381 and ATCC 33277 was significantly lower (23% and 32%, respectively) than with Porphyromonas gingivalis PK1924.

For the identification of Porphyromonas gingivalis galactose cell surface components as receptors for coaggregation with fusobacteria,
Table 1. Coaggregation of Fusobacterium nucleatum PK 1594 and Porphyromonas gingivalis strains PK 1924, ATCC 53978, 381 and ATCC 33277 in the presence of monosaccharides or EDTA

<table>
<thead>
<tr>
<th>P. gingivalis strain</th>
<th>Inhibitor addition</th>
<th>Coaggregation score</th>
<th>F. nucleatum binding (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK 1924</td>
<td>None</td>
<td>4+</td>
<td>19530 ± 1180</td>
</tr>
<tr>
<td></td>
<td>Gal (10 mM)</td>
<td>0</td>
<td>4290 ± 630</td>
</tr>
<tr>
<td></td>
<td>Glc (20 mM)</td>
<td>4+</td>
<td>18952 ± 1424</td>
</tr>
<tr>
<td></td>
<td>EDTA (10 mM)</td>
<td>0</td>
<td>3230 ± 423</td>
</tr>
<tr>
<td></td>
<td>CB without MgCl2</td>
<td>4+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CB without CaCl2</td>
<td>4+</td>
<td>ND</td>
</tr>
<tr>
<td>ATCC 53978</td>
<td>None</td>
<td>0</td>
<td>3016 ± 176</td>
</tr>
<tr>
<td>W 381</td>
<td>None</td>
<td>Autoaggregation</td>
<td>4638 ± 688</td>
</tr>
<tr>
<td>ATCC 33277</td>
<td>None</td>
<td>Autoaggregation</td>
<td>6390 ± 658</td>
</tr>
</tbody>
</table>

*Final inhibitor concentration.
†The coaggregation score was determined by the visual coaggregation assay.
‡Binding of Fusobacterium nucleatum to different Porphyromonas gingivalis strains is expressed as radioactivity accreted to the wells (dpm). The data are representative of three independent experiments.
ND, not determined; CB, coaggregation buffer.

Table 2. Binding of glycine-max lectin–peroxidase and Fusobacterium nucleatum PK 1594 to CPSs and LPSs from different Porphyromonas gingivalis serotypes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Glycine-max binding (OD)*</th>
<th>F. nucleatum binding (d.p.m.)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.143 ± 0.03</td>
<td>231 ± 24</td>
</tr>
<tr>
<td>CPS PK 1924</td>
<td>1.449 ± 0.17</td>
<td>24878 ± 2539</td>
</tr>
<tr>
<td>LPS PK 1924</td>
<td>0.648 ± 0.05</td>
<td>11183 ± 1318</td>
</tr>
<tr>
<td>CPS ATCC 53978</td>
<td>0.098 ± 0.02</td>
<td>857 ± 154</td>
</tr>
<tr>
<td>LPS ATCC 53978</td>
<td>0.086 ± 0.01</td>
<td>2130 ± 323</td>
</tr>
<tr>
<td>CPS W50 (K1)</td>
<td>0.132 ± 0.006</td>
<td>974 ± 144</td>
</tr>
<tr>
<td>CPS HG184 (K2)†</td>
<td>0.102 ± 0.01</td>
<td>168 ± 40</td>
</tr>
<tr>
<td>CPS A7A1-28 (K3)†</td>
<td>0.130 ± 0.01</td>
<td>241 ± 50</td>
</tr>
<tr>
<td>CPS 49417 (K4)†</td>
<td>0.087 ± 0.01</td>
<td>186 ± 27</td>
</tr>
<tr>
<td>CPS HG 1690 (K5)†</td>
<td>1.006 ± 0.05</td>
<td>28588 ± 3418</td>
</tr>
<tr>
<td>CPS HG1691 (K6)†</td>
<td>0.204 ± 0.006</td>
<td>27013 ± 1260</td>
</tr>
</tbody>
</table>

The data are representative of three independent experiments.
*Binding of Glycine–Max lectin to the six different Porphyromonas gingivalis K serotypes CPSs and LPSs from P. gingivalis PK 1924 and ATCC 53978 was determined by enzyme linked immunosorbent assay.
†Binding of Fusobacterium nucleatum to different CPSs and LPSs is expressed as radioactivity accreted to the wells (d.p.m.).
‡A kind gift from Dr Robert Schifferle, School of Dental Medicine, State of New York, Buffalo, USA.

P. gingivalis CPS and LPS from PK1924 and ATCC 53978 strains were extracted and purified by gel filtration (Schifferle et al., 1989; Choi et al., 1998). Table 2 shows that the purified P. gingivalis PK 1924 CPS and LPS bind F. nucleatum cells when compared to control wells (without ligand), or P. gingivalis ATCC 53978 CPS and LPS. Binding of fusobacteria to P. gingivalis CPS was significantly (P < 0.05) higher than to LPS. Fusobacterial cells were also found to bind to CPS from reference strains HG 1690 (serotype K5) and HG 1691 (serotype K6), when the six reference capsular polysaccharides (K1 to K6, strains W50, HG184, A7A1-28, 49417, HG1690 and HG1691, respectively) were tested.

The presence of GalNAc residues in the P. gingivalis polysaccharides was evaluated by the reaction of the glycine-max lectin with the purified compounds. Table 2 shows that CPS and LPS of P. gingivalis PK1924 (serotype K5) bind to the lectin. CPS from P. gingivalis HG 1690, that also belongs to the K5 serotype, reacted with the lectin.

To study the interaction between the F. nucleatum lectin and the CPS and LPS of P. gingivalis PK1924 the effect of a range of concentrations of different saccharides on binding of F. nucleatum to P. gingivalis PK 1924 CPS and LPS was tested. For each compound examined, inhibition curves were constructed to estimate the concentration of each carbohydrate that inhibited binding of F. nucleatum to P. gingivalis PK 1924 CPS and LPS by 50% (I₅₀). Table 3 shows the I₅₀ for the different carbohydrates and compares these values to the I₅₀ obtained by the inhibition of the same carbohydrates to the binding of F. nucleatum to the A. actinomycetemcomitans Y4 LPS (Rosen et al., 2003). The best inhibitors of binding of the F. nucleatum lectin to the CPS and LPS of P. gingivalis PK 1924 were Gal, lactose and...
related compounds with a free and axial hydroxyl group at position 4 and free or equatorial hydroxyl groups at positions 3 and 6 of α-Gal, similar to the observations reported with the Y4 LPS receptor. (Rosen et al., 2003).

Inhibition studies on binding of F. nucleatum to P. gingivalis serotype K6 CPS with different saccharides showed a similar inhibition pattern to the binding of F. nucleatum to the serotype K5 CPS (Table 3). EDTA (10 mM) inhibited binding of fusobacteria to PK 1924 LPS, PK 1924 CPS and HG 1691 CPS by 75%, 94% and 90%, respectively.

The P. gingivalis PK 1924 CPS was tested for its ability to inhibit the binding of F. nucleatum PK 1594 to P. gingivalis PK 1924 cells. LPS from Actinobacillus actinomycetemcomitans ATCC 29523, P. gingivalis ATCC 53978 and Escherichia coli 026 : B6, were used as controls. As shown in Fig. 1, P. gingivalis PK 1924 CPS and LPS by a series of saccharides inhibited binding of fusobacteria to PK 1924 LPS, PK 1924 CPS and HG 1691 CPS by a series of 3 independent experiments.

In order to evaluate if serotype K5 and serotype K6 CPS interact with the F. nucleatum cells through a similar mechanism, competitive binding studies were performed. Figure 3 shows that K6 CPS inhibits binding of the fusobacterial cells to both serotype K5 CPSs (PK1924 and GH1690) to a similar extent, in a concentration dependent manner.

**Discussion**

The findings of this study strongly suggest that the CPS and LPS of Porphyromonas gingivalis PK 1924 (serotype K5) act as receptors for coaggregation with Fusobacterium nucleatum. This contention is supported by the ability of CPS and LPS from P. gingivalis PK 1924 to bind to F. nucleatum cells as well as the inhibitory effect of these bacterial outer membrane molecules on the binding of F. nucleatum to P. gingivalis cells. Inhibition studies on the binding of the fusobacterial lectin to the P. gingivalis PK 1924 CPS and LPS receptors (Table 3), indicate that the most efficient inhibitors are lactose, α-MeGal, Gal and their derivatives, all with an axial free hydroxyl group at position 4, and free equatorial hydroxyl groups at positions 3 and 6 of α-galactose. The fusobacteria lectin also depends on a metal
divalent ion for binding to the \textit{P. gingivalis} CPS and LPS receptors. These are also the requirements reported for binding the \textit{Actinobacillus actinomycetemcomitans} serotype b LPS to fusobacteria (Rosen et al., 2003).

The GalNAc residues found in both the CPS and LPS of \textit{P. gingivalis} serotype K5 probably enabled their recognition by the galactose binding lectin (Table 2). It has been previously shown that the glycine-max lectin bound to \(\alpha\) or \(\beta\) GalNAc residues with free hydroxyl groups at positions 3 and 6 (Zeng et al., 2000).

Table 2 shows that fusobacterial cells bind to K6 CPS, in addition to K5 CPS. Inhibition studies with different saccharides show that the carbohydrate and divalent cation requirements for the binding of both CPSs are very similar (Table 3). Furthermore, serotype K6 CPS was found to inhibit binding of \textit{F. nucleatum} cells to \textit{P. gingivalis} serotype K5 CPS (strains PK1924 and HG1690) (Fig. 3). These data suggest that binding of CPS of \textit{P. gingivalis} serotype K6 and K5 to \textit{F. nucleatum} PK 1594 might occur through the same fusobacterial galactose binding lectin. The results of the sugar inhibition assays (Table 3) together with the fact that glycine-max lectin did not bind to K6 CPS (Table 2) suggest that Gal, Tal or their derivatives (with the exception of GalNAc), which respond to the structural requirements of the fusobacterial lectin, might be the recognition sites for \textit{F. nucleatum} PK 1594 on the \textit{P. gingivalis} K6 CPS receptor.
Galacturonic and glucuronic acids were reported to form part of the P. gingivalis capsular polysaccharide (Schifferle et al., 1989; Farquharson et al., 2000). These acidic monosaccharides were not likely to be the binding receptors to the F. nucleatum lectin as they were unable to inhibit binding of F. nucleatum PK 1594 to the P. gingivalis CPS (Table 3). Therefore, substitution at position 6 of Gal seems to block its inhibitory activity, as observed with galacturonic acid and Gal-6-sulphate.

Porphyromonas gingivalis ATCC 53978 (serotype K1) is the only strain where the chemical structure of the O-PS moiety of the LPS has been studied (Farquharson et al., 2000; Paramanov et al., 2001). Our findings that P. gingivalis ATCC 53978 did not coaggregate with F. nucleatum PK 1594 may be related to the absence of free hydroxy groups at position 3 of α-Gal or α-GalNAc in the O-PS moiety which probably prevented the recognition of its LPS by the galactose binding lectin/s of fusobacteria. It was also shown that the CPS of this P. gingivalis strain was unable to bind F. nucleatum PK 1594 cells (Table 2).

The present report demonstrates that both CPS and LPS from P. gingivalis serotype K5 bind F. nucleatum cells and inhibit coaggregation between these two oral bacteria. Binding of F. nucleatum cells to CPS is significantly higher than to LPS (Table 2). Nevertheless, inhibition by LPS is better than by CPS (Figs 1 and 2). Inhibition of coaggregation in vitro, may be influenced by the different molecular organization or folding of the CPS and LPS present in the fluid state. Studies on the soluble conformation of capsular polysaccharides from different bacteria have suggested they are rather flexible molecules with a random coil conforma-

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### References


