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Binding of the periodontitis associated bacterium *Porphyromonas gingivalis* to glycoproteins from human epithelial cells

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Introduction: In the present study we examined the ability of the periodontal pathogen *Porphyromonas gingivalis* to adhere to glycoconjugates on intact cells and to protein preparations of epithelial cells (KB cells).
Methods: The KB cell protein preparation was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by Western blotting. The membranes were used in overlay assays with labeled *P. gingivalis*. Flow cytometry was used to analyze attachment of bacteria to intact KB cells.
Results: Glycoconjugate expression on the KB cells and in the protein preparation was confirmed. Binding was detected to several bands on the Western blots. Flow cytometry showed a distinct increase in fluorescence for strain FDC 381. Preincubation of the bacteria with mannose, fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine inhibited the binding to KB cells by approximately 30% whereas preincubation with *N*-acetylneuraminic acid reduced the binding by 60%.
Conclusion: These results indicate that carbohydrate structures are involved in the binding process of *P. gingivalis* to oral epithelial cells and that neuraminic acid plays a significant role in the adhesion process.

Key words: adhesion; glycoconjugates; glycoprotein; neuraminic acid; *Porphyromonas gingivalis*

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Periodontitis is a widespread disease that affects as many as 10–15% of the population (3, 13). The disease is initiated by colonization of the gingival crevice by pathogenic bacteria. Common bacteria in the non-healthy pocket are the anaerobic gram-negative *Actinobacillus* ssp., *Fusobacterium* ssp., *Campylobacter rectus*, and *Porphyromonas gingivalis*. Of these, the last is considered the most important in this context. Previous studies have shown that *P. gingivalis* has a large array of virulence factors, including the ability to adhere to and invade oral epithelial cells *in vitro* (7, 21), primary cultures of periodontal pocket epithelium (22), and also endothelial cells from coronary vessels and human umbilical vein (5, 6). Invasion is a way for the bacterium to circumvent the host immune defense and obtain the opportunity to multiply and further invade the target tissue. In addition to the ability to attach to tissue, *P. gingivalis* also produces a number of proteolytic enzymes that contribute to its virulence. These enzymes can impede the function of T cells (15), for example, and thereby contribute to sheltering the bacteria from the immune system. *P. gingivalis* therefore possess characteristics that promote the adhesion and invasion of the bacteria and at the same time inactivate the host defense and repair system.

The ability of several different bacteria to use carbohydrates as receptors for binding to eukaryotic cells is well documented and has been shown for *Helicobacter pylori* (2, 24), *Escherichia coli* (25), and *Streptococcus suis* (9). Our aim is to examine the possibility that *P. gingivalis* uses carbohydrate structures for adhesion to cells. The binding of *P. gingivalis* to cell membrane extracts has been investigated (1). It was shown that sialic acid residues and glucuronic acid might play a role in the adherence of this bacterium to oral epithelial cells. Furthermore, we have shown that *P. gingivalis* can bind to both acidic and non-acidic glycolipid fractions from a variety of organs of human and porcine origin, with a preference for sulfated glycolipids (11). We have also shown a preference for *P. gingivalis* to bind to cerebroside with non-hydroxy fatty acid, as compared with gastrointestinal bacteria, which prefer hydroxy fatty acid (10). The aim of this present study was to investigate the ability of the periodontal pathogen *P. gingivalis* to adhere to glycoconjugates on intact cells and to glycoprotein preparations of epithelial cells.

Materials and methods

Bacterial strains and culture conditions

The *P. gingivalis* strain used was the wild-type strain FDC 381 (adhesive and invasive; Forsyth Dental Center, Boston, MA). The bacteria were cultivated on *Brucella* agar plates (BBL Microbiology Systems, Cockeysville, MD) under anaerobic conditions (95% H_2 and 5% CO_2) at 37°C for 24 h. *E. coli* were cultured on agar plates under aerobic conditions at 37°C for 12 h.

Cells and culture conditions

A fast growing cell line, KB (ATCC CCL 17), originating from epithelial carcinoma cells was used for the studies. The cells were cultured in Dulbeccos's modified Eagle's medium supplemented with 200 mM L-glutamine, 10% fetal calf serum, and 100 mg/ml penicillin–streptomycin. Cells used for protein preparation were cultured for 2 days, fresh medium was added, and the cells were harvested the next day. Cells used for flow cytometry were cultured for 3 days before harvest. At harvesting, the cells were washed three times in phosphate-buffered saline (PBS) before they were carefully loosened from the plastic with a cell scraper.

Bacterial labeling

Biotin labeling was performed according to Falk et al. (8). Briefly, the bacteria were recovered from the culture plates and washed once in cold PBS and once in carbonate buffer (0.2 M $NaHCO_3$, pH 8.3). Bacteria were suspended in carbonate buffer and the concentration was calculated by measuring the optical density at 550 nm. Two milliliters of bacteria at 10^{10} bacteria/ml were labeled with 10 µl of 10 mg/ml biotin–*N*-hydroxysuccinimide ester (Sigma-Aldrich, St Louis, MO) in dimethylsulfoxide (Sigma-Aldrich) on ice for 1 h in the dark with occasional stirring. Labeled bacteria were washed twice in PBS-Tween (0.05% Tween-20 in PBS) and resuspended in 1 ml of the buffer. The bacteria were stored in aliquots at −20°C.

Fluorescein isothiocyanate (FITC) labeling was performed as described by Korhonen et al. (17). The bacteria were harvested and washed twice in cold PBS. The concentration was measured as above and 8×10^8 bacteria were suspended in 2 ml of 0.1 M Na_2CO_3, pH 9.0 containing 0.9% (weight/volume) NaCl and 75 µg FITC per ml (Sigma-Aldrich). The bacteria were incubated on ice for 45 min in the dark with occasional stirring. Labeled bacteria were washed twice in PBS-Tween, resuspended in 1 ml PBS, and stored as above.

Flow cytometry

Washed KB cells, suspended at a concentration of 10^6 cells/ml, were incubated with FITC-labeled bacteria at a concentration of 10^9 bacteria/ml for 80 min at 4°C. The cells were washed twice in PBS before being counted in the flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ); 10,000 events were registered. Possible unbound bacteria and cell debris were excluded from analysis by the threshold setting.

Blocking experiments with soluble saccharides (Sigma-Aldrich) were performed by incubation with 100 mM mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, or *N*-acetylneuraminic acid, respectively. The bacteria were preincubated with the saccharide for 15 min before addition of the cells. All saccharides were dissolved in PBS except for *N*-acetylneuraminic acid, which was made up in TBS (20 mM Tris, 500 mM NaCl pH 7.5) (20). Bacteria and cells were incubated as above.

Preparation of protein

KB cells cultured for 3 days were harvested and suspended at 5×10^6 cells/ml in ice-cold PBS and centrifuged at 500 ***g*** at 4°C. The washing step was repeated once. The cells were then resuspended in lysis buffer (300 mM NaCl, 50 mM Tris–HCl, 0.5% Triton X-100, pH 7.6) with protease inhibitors – Complete™ protease inhibitor (Roche Diagnostics, Basel, Switzerland) – at 10^8 cells/ml and incubated on ice for 45 min with occasional stirring. The lyzed cells were centrifuged at approximately 10,000 ***g*** on an Eppendorf centrifuge for 15 min at 4°C. The supernatant and pellet were separated and stored at −20°C.

Bacterial overlay analysis

The protein preparation supernatant was separated on a 10–20% Tris–HCl polyacrylamide gel (BioRad Laboratories, Hercules, CA) and blotted to nitrocellulose membrane (BioRad). The membrane was blocked in casein-TBS (BioRad Laboratories, Hercules, CA) for 1 h, washed three times for 5 min each time in TBS-Tween (0.05% Tween-20 in TBS) and once in TBS before incubation with biotin-labeled bacteria at a concentration of 10^7/ml on an orbital shaker at slow speed for 3½ h. The membranes were washed four times in TBS-Tween and twice in TBS before incubation with ExtrAvidin peroxidase (Sigma-Aldrich) at 1 : 3000 for 30 min. Washing was performed three times in TBS-Tween and once in TBS before the substrate, SigmaFAST DAB (Sigma-Aldrich), was added. After development of the colored product the membranes were rinsed in water and allowed to dry at room temperature.

Glycan detection

KB protein underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was blotted to a nitrocellulose membrane. The membrane was then used for glycan detection using the digoxigenin (DIG) glycan detection kit (Roche Diagnostics) as recommended by the manufacturer. Briefly, washed membranes were incubated in 10 mM sodium metaperiodate in 0.1 M sodium acetate buffer, pH 5.5 for 20 min at room temperature. After washing in PBS the filters were incubated with 1 µl DIG-*O*-3-succinyl-ϵ-aminocaproic acid hydrazide in 5 ml sodium acetate buffer, pH 5.5 for 1 h before they were washed in TBS and

incubated for at least 30 min in blocking solution. Washing in TBS was performed before incubation in anti-DIG-alkaline phosphatase in TBS for 1 h. After a final washing in TBS the membranes were dried in room temperature.

The protein extract was also treated with 100 mU/ml sialidase for 1 h at 37°C before electrophoresis and blotting. Bacterial overlay and glycan detection with sialic-acid-specific oxidation was then performed.

Wheat germ lectin column affinity chromatography

HiTrap wheat germ lectin column, 1 ml, (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was washed and equilibrated as recommended by the manufacturer; 1.5 ml of the protein preparation supernatant was diluted to 3 ml in TBS and applied slowly to the column. The column with sample was incubated for 1 h at 4°C before washing with 10 ml TBS. The bound sample was eluted with 0.5 M *N*-acetylglucosamine (Sigma-Aldrich) in TBS.

Results

Glycan detection and bacterial overlay assay

To verify that the cultured cells expressed glycoconjugates and to assure that the carbohydrates were not lost during the preparation process of the proteins, we used the DIG-glycan detection kit on electrophoresis blots. The result was positive for the majority of bands seen on the gel.

The nitrocellulose membranes, with SDS–PAGE separated and blotted KB cell membrane proteins, were also incubated with biotin-labeled *P. gingivalis*. Binding was detected to several of the bands (Fig. 1).

After treatment of the protein extract with sialidase, the DIG protocol verified a reduction of sialic acid on the glycoproteins on the blot. However, the *P. gingivalis* binding was only marginally reduced. (Fig. 2).

Flow cytometry

The binding of *P. gingivalis* to intact viable cells was analyzed by flow cytometry. Cells incubated with *P. gingivalis* showed a distinct increase in fluorescence (Fig. 3) compared to the autofluorescence caused by the cells alone.

For comparison, FITC-labeled *E. coli* were incubated with KB cells. This bacterium is not known to bind to KB cells and the flow cytometry histogram showed, as expected, only a slight shift to the right of the autofluorescence of the KB cells (Fig. 3).

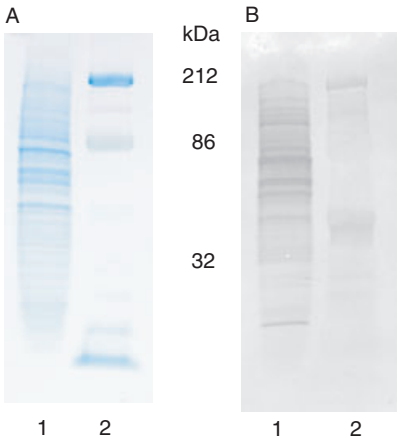

Fig. 1. Analysis of protein extract from cultured KB cells. The samples were separated by gel electrophoresis on a 10–20% Tris–HCl polyacrylamide gel. The gel was stained with BioSafe Coomassie (A) and the proteins were blotted to nitrocellulose membrane. Bacterial overlay with biotin-labeled *Porphyromonas gingivalis* FDC 381 was performed on the membrane after casein blocking of unspecific binding (B). Lane 1 is the protein extract and lane 2 is a molecular weight marker (Kaleidoscope). Molecular weight is indicated in thousands.

To investigate the importance of saccharides for the binding of *P. gingivalis* to epithelial cells, the bacteria were incubated together with soluble saccharides before mixing with the cells. The results are shown in Fig. 4. Neuraminic acid inhibited the binding of *P. gingivalis* to KB cells by approximately 60%, whereas the other saccharides tested (mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine) inhibited binding to about 30%.

The analyses were performed with and without 0.1 mM $CaCl_2$. No significant difference could be detected. The interactions of viridans group streptococci to galactose-containing and *N*-acetylgalactosamine-containing receptors have been shown to be calcium dependent, whereas sialic acid-containing receptor interactions were calcium independent (23).

Discussion

Animal cells carry glycoconjugates on their surface. The same individual expresses different cell surface glycoconjugates in different cell types and the expression varies in the same cell during the differentiation process (4, 19). Cells that are less differentiated express short and unbranched carbohydrates but more differentiated cells express longer and more complex carbohydrate chains. Therefore, the expression of glycoconjugate on

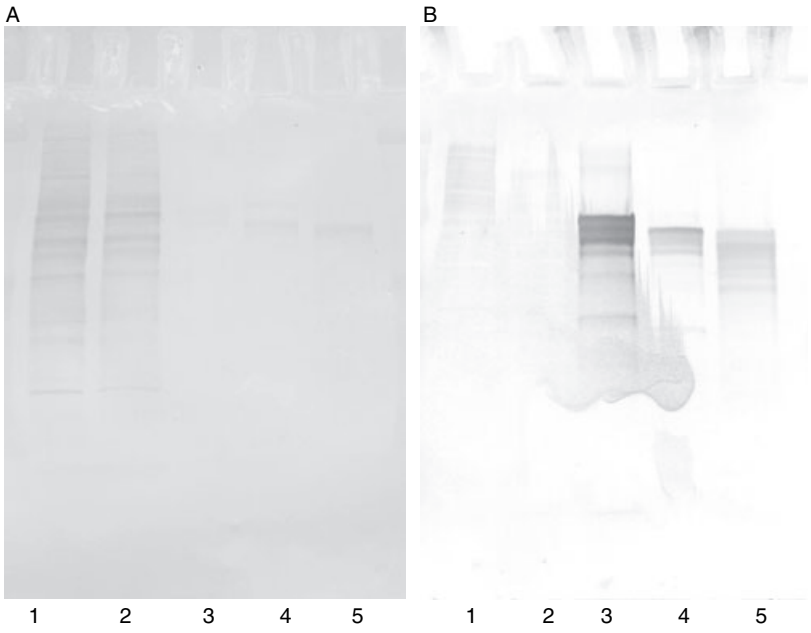

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot (performed as in Fig. 1) analyses of native and sialidase treated glycoproteins. Lane 1, protein preparation of KB cells; lane 2, protein preparation of KB cells after sialidase treatment; lane 3, fetuin; lane 4, fetuin after sialidase treatment; lane 5, asialofetuin. The membranes were (A) subjected to bacterial overlay (performed as in Fig. 1) and (B) used for glycan detection by the digoxygenin glycan detection kit.

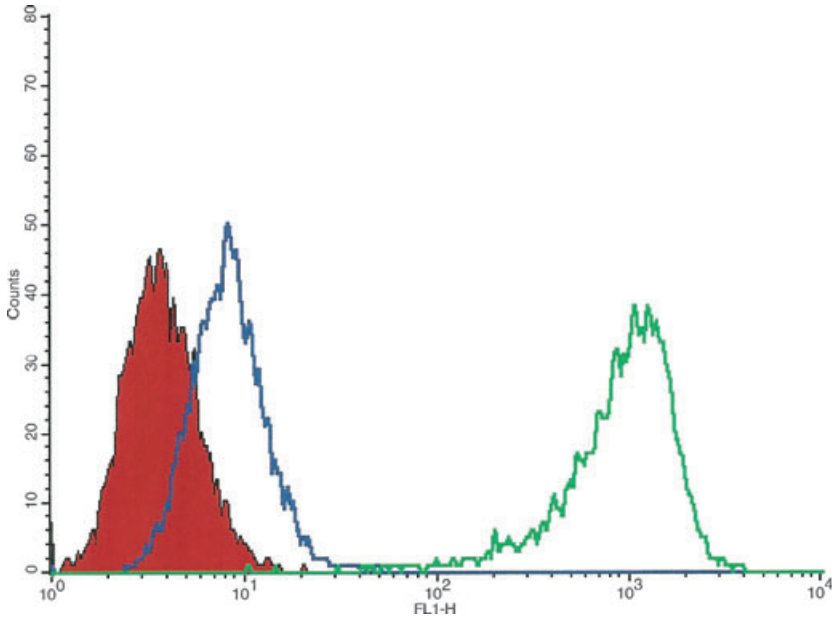

Fig. 3. Flow cytometry histogram showing adhesion of fluorescein isothiocyanate-labeled bacteria to viable KB cells. The cells were incubated with the labeled bacteria for 80 min before unbound bacteria were washed off. The red curve shows the autofluorescence caused by the cells alone. *Porphyromonas gingivalis* adhere to the cells; this results in an increased fluorescence (green curve), whereas *Escherichia coli* are non-adherent and so are lost during the wash (blue curve).

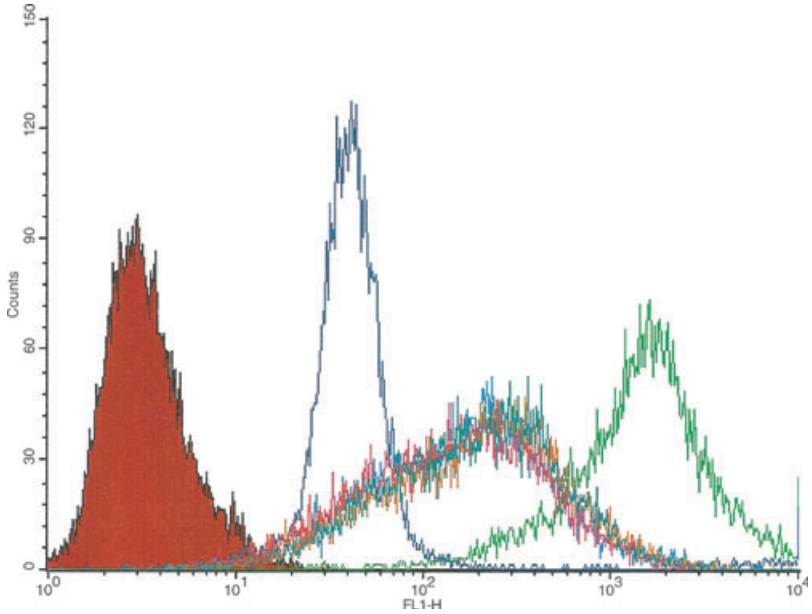

Fig. 4. Flow cytometry histogram showing saccharide inhibition of *Porphyromonas gingivalis* adhesion to viable KB cells. Fluorescein isothiocyanate-labeled FDC 381 were incubated with KB cells for 80 min (green curve). Bacteria were incubated with soluble saccharide for 15 min before incubation with the cells. Blue curve, 100 mM *N*-acetylneuraminic acid; multicoloured curve, 100 mM mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, respectively. The red curve shows the autofluorescence caused by the cells alone.

the surface of cells in culture can be expected to vary by time after passage of the cells. During the exponential phase of growth it is conceivable that the cells have less need to express cell surface molecules that are not necessary for proliferation. We chose to culture the cells for 3 days before harvest. By this time, they were confluent and the growth had reached a plateau. To affect the cell surface glycoproteins and proteoglycans as little as possible we did not use enzymatic treatment to dissociate the cells.

The method for overlay analysis with bacteria and glycoconjugates bound to a solid surface, was first developed by Karlsson (14). He used glycolipids that were separated by thin layer chromatography according to the size of the carbohydrate part. Using this method only the lipid-bound carbohydrates are analyzed, all other structures are removed during the preparation process. When the overlay method is used on Western blots, it is only protein-bound carbohydrates that are analyzed. Using electrophoresis it is not the size of the carbohydrate part that is the basis for separation but the glycoproteins are separated according to the size of the protein part. Another difference between lipid-linked and protein-linked carbohydrates is that only one carbohydrate chain is bound to each lipid molecule whereas several chains of varying carbohydrate sequences and sizes can be linked to one protein molecule. When glycoprotein extracts undergo electrophoresis and blotting, structures of both carbohydrate and protein nature are exposed. On the intact cells, the bacteria cannot normally access all these structures. The structures are also exposed in a different way *in vitro*; they can be more elongated or differently folded when in solution so the bacteria can access parts of the molecules that are not exposed *in vivo*. This illustrates the need for different analytical methods for use in binding studies. Our overlay analyses show that *P. gingivalis* adheres to several different bands on the gel. This suggests that the structure (or structures) detected by *P. gingivalis* is present on many different proteins with different size and different net charge, which is shown by the fact that separation by molecular weight and by ion exchange could not distinguish any specific group of glycoproteins where binding was more prominent. This indicates that the structure recognized by the bacteria is a core structure, which constitutes a base for longer structures. *P. gingivalis* produces enzymes that can hydrolyze proteins and thus expose normally hidden epitopes (16). The labeled bacteria were stored at −20°C for several months without impairment of the binding. This is of great advantage because it minimizes the variability between experiments performed on different occasions and allows better comparison between analyses.

Sialidase treatment of the protein extract reduced the quantity of sialidase molecules

presented on the glucoproteins as verified by the DIG protocol. The *P. gingivalis* binding was only marginally reduced. This might be explained by exposure of different binding epitopes after enzyme treatment or by a higher sensitivity for the epitopes by *P. gingivalis* compared to the DIG detection. Whole bacteria were used for the adhesion studies. Earlier studies on *P. gingivalis* adhesion have mainly dealt with fimbriae binding to membranes. The advantage of using whole bacteria is the possibility of detecting other adhesion mechanisms than those used by fimbriae. In the overlay assay the nature of the binding was not determined; both carbohydrate and protein binding are likely to occur.

In the fluorescence-activated cell sorting analyses, neuraminic acid was shown to decrease binding of *P. gingivalis* to KB cells. Similar findings have been made for other bacteria (12, 18). In flow cytometry, intact KB cells are used; this represents a more *in vivo*-like appearance of the structures because surrounding structures, which can influence the binding, are present. The glycoconjugates detected by fluorescence-activated cell sorting can be linked to either lipid or protein, but bacterial binding is restricted to molecules presented on the cell surface. In a study of membrane extracts there is a risk that the extracts form inverted micelles and so the lipid or protein part is exposed instead of the carbohydrates on the cell surface. Part of the epitopes can be hidden or molecules that are not normally accessible for the bacteria can be exposed. Of course, we cannot rule out the possibility that bacteria have also invaded cells and that some increase of the fluorescence is the result of invading bacteria.

In conclusion, binding of *P. gingivalis* to eukaryotic cells may be the result of several different mechanisms. The binding to carbohydrate can be to structures carried by both lipids and proteins. The structures are present on several different proteins and it is probably a carbohydrate core structure. Binding is partly dependent on the presence of sialic acid.

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