Structures involved in the interaction of *Porphyromonas gingivalis* fimbriae and human lactoferrin

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Abstract The ability of laboratory and clinical strains of *Porphyromonas gingivalis* to bind lactoferrin has been assessed (FEMS Immunology and Medical Microbiology, 1996, 14, 135–143). Relative binding for *P. gingivalis* to lactoferrin varies among strains from 3.78 to 26.62%. We also observed that fimbriated strains of *P. gingivalis* bind more strongly to lactoferrin as compared to nonfimbriated strains of *P. gingivalis*. This observation led us to study fimbrial interaction with human lactoferrin and the fine structure of these interactions. Binding of iodinated purified fimbriae was studied using an overlay assay. Iodinated fimbriae bind specifically and strongly to human lactoferrin. When various sugars were used to inhibit binding, only *N*-acetylgalactosamine and fucose were inhibitory. To confirm further that oligosaccharide of lactoferrin is involved in the interaction, lactoferrin was chemically deglycosylated, and fimbriae failed to bind deglycosylated lactoferrin. Antifimbriae, as well as four antipeptide antibodies against different regions of the *P. gingivalis* fimbrillin, were used to inhibit the interaction. Antipeptide E, directed against amino acids 81–98 (AAGLIMTAEPKTIVLKAG-C), was found to be the most effective inhibitor for the lactoferrin-fimbriae interaction. These results suggest that the binding of *P. gingivalis* cells to lactoferrin is lectin like, directed to a oligosaccharide of lactoferrin. Furthermore, these studies suggest that the region of fimbriae that binds to lactoferrin is the N-terminus of the molecule. It is likely that binding of lactoferrin to *P. gingivalis* cells results in antimicrobial activity directed against these cells by virtue of its ability to deprive the bacterial cell of needed iron.

Key words: Lactoferrin; Periodontal disease; *Porphyromonas gingivalis* fimbria; Iron complexing capacity

1. Introduction

Porphyromonas gingivalis is a black-pigmented anaerobe associated with the progression of periodontal disease [1]. Haemin, lactoferrin, and transferrin are known to be present at periodontally diseased sites and *P. gingivalis* can bind and obtain sequestered iron from these proteins [2].

Lactoferrin, a mammalian iron binding, acute-phase protein, belongs to the innate immune system, and is present in many external secretions [3], as well as in the specific granules of neutrophils. Lactoferrin is also found in the gingival crevicular fluid and concentrations are dependent on the severity of periodontal inflammation [4]. The antimicrobial properties of lactoferrin include bacteriostatic and bactericidal effects on a variety of microbial pathogens [5–7]. Lactoferrin inhibits the adhesion of certain bacteria to epithelial cells [8,9], as well as the adhesion of putative periodontal pathogens to human plasma proteins, connective tissue components, epithelial cells and fibroblasts [10–12].

Various studies have shown that lactoferrin has strong bacteriostatic and bactericidal effects, as well as a role in regulating various components of the immune system [13]. Lactoferrin in gingival crevicular fluid may be from polymorphonuclear leucocytes which have infiltrated the gingival sulcus during the progression of periodontal disease. The antimicrobial activity of lactoferrin resides in its iron complexing capacity, depriving bacteria of this important ion. The binding of lactoferrin to the cell surface is a preliminary step in its antibacterial activity. Recent studies show that the development of periodontal disease is associated with increased levels of proteolytic activity, some of microbial origin [14–17]. It seems possible that some *P. gingivalis* adhesins might bind to lactoferrin, and proteases produced by *P. gingivalis* will degrade lactoferrin or cleave N-terminal, thereby destroying its antimicrobial activity [18]. Therefore, the aim of this study was to examine the role of *P. gingivalis* fimbriae in the binding of lactoferrin. The binding and degradation of lactoferrin by *P. gingivalis* may be a virulence factor in the progression of periodontal disease.

2. Materials and methods

2.1. Bacterial culture conditions

P. gingivalis strain 2561 was grown in half strength (18 mg/ml) brain heart infusion broth (Difco) supplemented with 5 mg of yeast extract per ml and buffered at pH 7.4. Cells were incubated for 2 days in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2).

2.2. Fimbrial preparation and antisera production

Fimbriae were purified by the method of Sojar et al. [19] and purity was confirmed by SDS-polyacrylamide gel stained with silver. Polyclonal rabbit antibodies to purified fimbriae, as well as peptide were produced as described earlier [20].

2.3. Iodination of purified fimbriae

Purified fimbriae were iodinated using the chloramine T method [21] with a slight modification. Ten micrograms of purified fimbriae were labelled using 0.5 mCi of sodium [^{125}I]iodide (Amersham Corp.) in 0.5 M phosphate buffer, pH 7.2, in the presence of 10 μl of chloramine T (mg/ml) for 60 s and iodination was terminated by adding 20 μl of sodium metabisulfite (2 mg/ml). After termination, 100 μl of phosphate buffer containing 10% sucrose and 10% potassium iodide was added and the mixture was loaded onto Sephadex G75 (1×30 cm) saturated with 1% bovine serum albumin (BSA) to prevent nonspecific binding. The column was extensively washed after saturating

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with BSA. The iodinated fimbriae peak was collected without carrier protein.

2.4. Chemical deglycosylation of lactoferrin

Lactoferrin was chemically deglycosylated by the method of Sojar and Bahl [22]. In brief, protein was incubated with trifluoromethanesulfonic acid (TFMS) on ice for 2 h, followed by neutralization of the acid with aqueous pyridine at −20°C. The neutralized reaction mixture was dialyzed with several changes of 0.01% ammonium bicarbonate at pH 7.0.

2.5. Analytical methods

SDS-PAGE was performed by the method of Laemmli [23] using Tris-glycine running buffer. Gels were stained with Coomassie brilliant blue stain. The protein content was determined by the Bradford method [24] using bovine serum albumin (BSA) as the standard.

2.6. Overlay assay

Overlay assay samples were prepared for SDS-PAGE in 0.125 M Tris-Cl, buffer pH 6.8, containing 1% SDS, 10% glycerol, 0.001% bromophenol blue with 5% β-mercaptoethanol at room temperature for 15 min. Samples were run on 10% SDS-PAGE using Tris-glycine as running buffer and transferred to Immobilon P (polyvinylidene difluoride) membrane by Western transfer with a semidry transfer system (Semi-phore TE 77: Hoefer Scientific Instruments, CA) at a constant current of 180 mA for 1 h. After transfer, unoccupied sites of the membrane were blocked with 1% Tween 20 for 1 h at room temperature. After blocking, the membrane was incubated with iodinated fimbriae at room temperature overnight. After extensive washing with TBS, bound fimbriae were determined by autoradiography.

2.7. Inhibition by sugars

For sugar inhibition studies, a 10% preparative gel was run and transferred to the membrane, blocked with 1% Tween 20 as mentioned above and cut in equal widths of 1 cm. Each strip was incubated with 50 000 cpm of iodinated fimbriae in the presence and absence of 50 μg of glucose, galactose, lactose, fucose, rhamnose, sialic acid *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, and maltose. On autoradiography all strips showed bands. The same strips were counted in a Beckman gamma counter, model 5500, for quantitation.

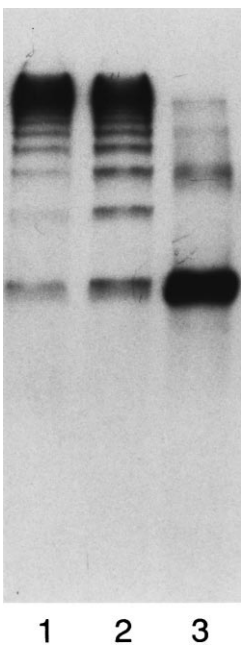

Fig. 1. Autoradiogram of iodinated purified fimbriae. Samples were run on SDS-10% polyacrylamide gels. After fixing, gel was washed extensively with distilled water and fixed in 2% glycerol. After fixing in glycerol, the gel was dried and exposed to X-ray film. Lane 1, 10 000 cpm of iodinated fimbriae in 2×sample buffer (0.25 M Tris base, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue, 10% β-mercaptoethanol), for 10 min. Lane 2, 10 000 cpm of iodinated fimbriae in 2×sample buffer heated at 80°C for 10 min. Lane 3, 10 000 cpm of iodinated fimbriae in 2×sample buffer heated at 100°C for 10 min.

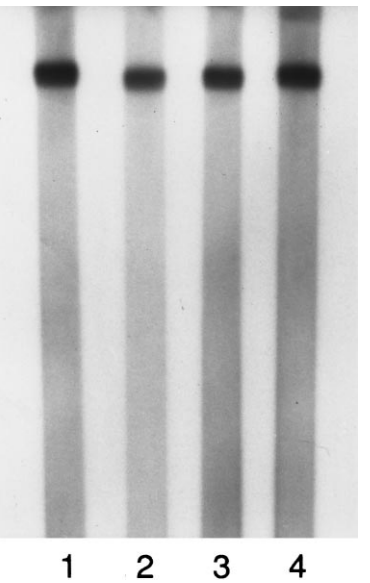

Fig. 2. Overlay assay with iodinated fimbriae. Lactoferrin samples were run on SDS-10% polyacrylamide gels and transferred to immobilon. After transfer, the membrane was blocked with 1% Tween 20 and incubated with iodinated fimbriae overnight at room temperature. Membrane was washed extensively with Tris-saline buffer. After washing and drying, the membrane was exposed to X-ray film. Lane 1, 5 μg of lactoferrin. Lane 2, 10 μg of lactoferrin. Lane 3, 15 μg of lactoferrin. Lane 4, 20 μg of lactoferrin.

2.8. Immunogold labelling

P. gingivalis strain 2561 cells were washed with PBS, resuspended, and transferred to nickel grids coated with Formvar film and air dried as described above. The cells were then incubated with 10 μl of rabbit polyclonal antibodies raised against peptide E (1:500 in PBS containing 1% BSA) at 37°C for 1 h. After washing four to five times with PBS, the cells were incubated for 30 min with sheep antirabbit IgG conjugated with 5 nm gold particles (1:20, AuroProbe EM, Amersham) at 37°C for 30 min. The cells were rinsed twice with PBS and negatively stained with 2% (w/v) uranyl acetate for 1 min. The fixed and stained cells were examined and photographed with an Hitach H-600 electron microscope operating at 75 kV.

3. Results and discussion

The binding of lactoferrin to purified fimbriae was determined by dot blot assay using an overlay assay. Purified fimbriae were iodinated using the chloramine T method and the integrity of labelled fimbriae was confirmed by autoradiography (Fig. 1). Purified fimbriae bound to lactoferrin very specifically (Fig. 2). Further, when binding of iodinated fimbriae to lactoferrin was checked in the presence of various sugars as described in Section 2, only *N*-acetylgalactosamine and fucose were found to be inhibitory. On autoradiography all strips showed +ve bands, but when the same strips were counted in a gamma counter, binding in the presence of fucose and *N*-acetylgalactosamine was found to be reduced by 60 and 70%, respectively, as compared to binding without sugar (data not shown).

In order to confirm that oligosaccharide of lactoferrin is involved in fimbriae interaction, lactoferrin was chemically deglycosylated and various concentrations of native and de-

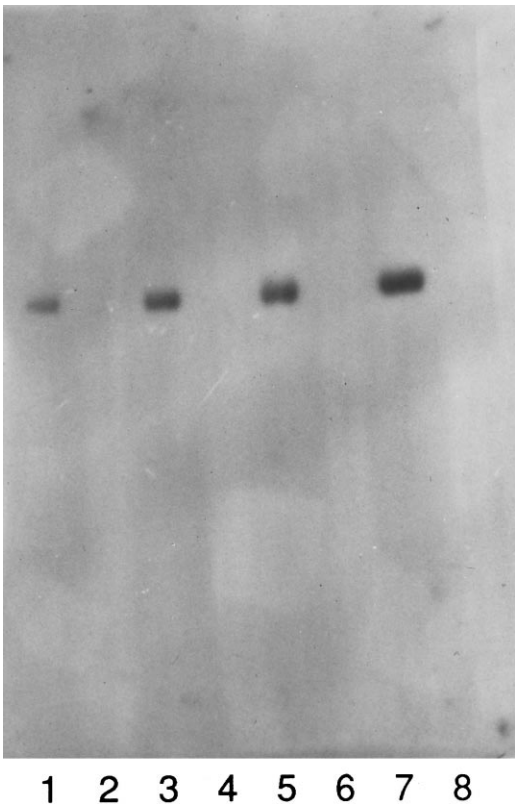

Fig. 3. Overlay assay with iodinated fimbriae. Lactoferrin and deglycosylated samples were run on SDS-10% polyacrylamide gels and transferred to immobilon. After transfer the membrane was blocked with 1% Tween 20 and incubated with iodinated fimbriae overnight at room temperature. Membrane was washed extensively with Tris-saline buffer. After washing and drying, the membrane was exposed to film. Lane 1, 2 μg of lactoferrin. Lane 2, 2 μg of deglycosylated lactoferrin. Lane 3, 4 μg of lactoferrin. Lane 4, 4 μg of deglycosylated lactoferrin. Lane 5, 6 μg of lactoferrin. Lane 6, 6 μg of deglycosylated lactoferrin. Lane 7, 8 μg of lactoferrin. Lane 8, 8 μg of deglycosylated lactoferrin.

glycosylated lactoferrin were run on SDS-PAGE and transferred to nitrocellulose. After blocking, the membrane was incubated with iodinated fimbriae and exposed for autoradiography. No binding to deglycosylated lactoferrin was observed (Fig. 3).

To determine which portion of the fimbriae is involved in lactoferrin interaction, four antipeptide antibodies corresponding to the residues 49–68 (VVMANTGAMELVGKTLAEVK-C), 69–90 (ALTTELTAENQEAAGLIMTAEP-C), 81–98 (AAGLIMTAEPKTIVLKAG-C), and 99–110 (KDYIGYSGTGEG-C), respectively, of the fimbrillin were used to inhibit the binding [20]. The choice of peptides for these studies was based on the secondary structure and hydrophilicity predictions according to the algorithms of Chou and Fasman [25]. Iodinated fimbriae were preincubated with antifimbriae and the respective antipeptide polyclonal antibodies for 2 h at room temperature prior to use for the overlay assay with lactoferrin, only antifimbriae polyclonal antibodies and antipeptide 81–98 were found to be inhibitory (data not shown). Further, to determine whether antipeptide 81–98 recognized the native fimbriae, whole cell was labelled by incubating with antipeptide 81–98 antibodies using an immunogold labelling technique (Fig. 4). No labelling was observed when normal rabbit sera were used as control (data not shown).

Fimbriae in particular have been suggested as playing an important role in facilitating the initial interaction between the bacteria and the host. Recently, Murakami et al. [26] showed that *P. gingivalis* fimbriae strongly induced TNF-α gene expression in the macrophages, and expression of TNF-α was inhibited by *N*-acetyl-D-galactosamine, but was not inhibited by other sugars. In this report we describe the potential binding of *P. gingivalis* fimbriae to lactoferrin. Our studies show that oligosaccharide of lactoferrin is involved in the interaction with *P. gingivalis* fimbriae. Amino terminal 81–98 of the fimbriae molecule may be, along with other domains, involved in lactoferrin interaction. Earlier studies [27] have shown that *P. gingivalis* cell surface proteases degrade the lactoferrin. *P. gingivalis* fimbriae may bind to lactoferrin and then be degraded by cell-associated proteases. This may provide protection to the cell against the effects of lactoferrin in periodontal sites and may be a possible virulence factor in disease. The ability of fimbriae to bind lactoferrin and more restricted specific residues involved in this interaction is currently being investigated. Intense research during the last decade has demonstrated several functions for lactoferrin in physiological and pathological conditions [28,29]. This leads us to believe that lactoferrin interaction with oral bacteria

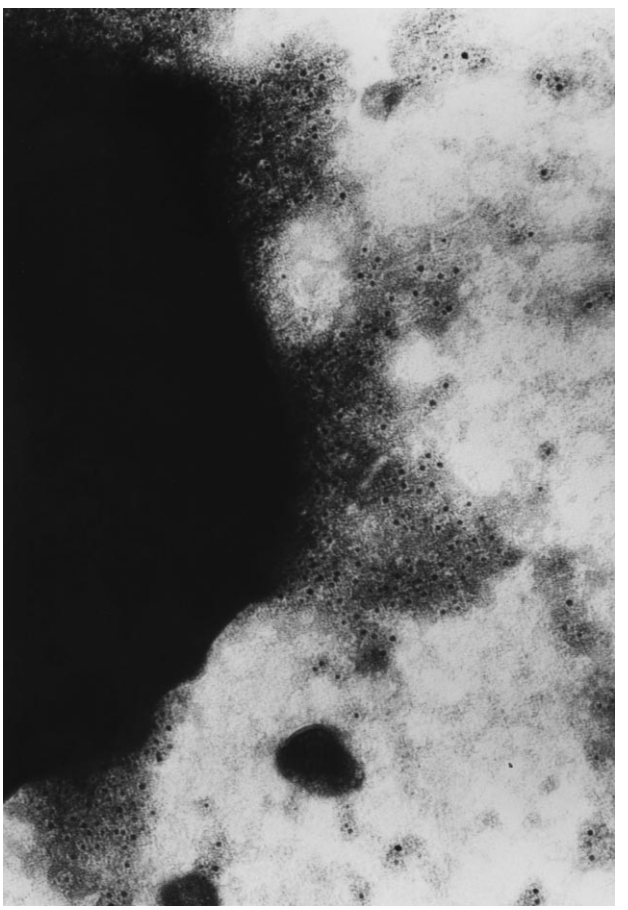

Fig. 4. Localization of fimbriae on whole cells, by immunogold electron microscopy. Cells were incubated with peptide E polyclonal antibodies, followed by 5 nm of colloidal gold-labelled goat antirabbit serum. Samples were prepared by negative staining with 2% uranyl acetate.

might be of significance for adhesion and colonization, and for development of the oral infectious processes.

Recent studies have shown binding of lactoferrin to cells of *P. gingivalis* and *P. intermedia* [30,31]. Further binding and degradation of lactoferrin by *P. gingivalis*, *P. intermedia*, and *P. nigrescens* were studied by Lillo et al. [32]. Their plots were nonlinear, giving a biphasic curve with two different slopes for all three species studied. An upwardly concave Scatchard plot could be due to binding site heterogeneity or negative co-operativity. Negative co-operativity usually occurs when oligomeric proteins are implicated in binding. Since fimbriae are oligomeric chains of fimbrillin molecules with a mass of 43 kDa, they possibly may be involved in binding with lactoferrin. The presence of fimbriae on fimbriated strains of *P. gingivalis* may explain the higher binding to lactoferrin in fimbriated strains of *P. gingivalis*. Because Lillo et al. [32] used whole cells in their binding studies, they expected binding heterogeneity due to co-existence of a wide range of molecules on the Gram-negative surface. Their data suggest that there are two molecular populations with high and low binding affinities. They have suggested that lactoferrin binds to a high affinity receptor on *P. gingivalis*, *P. intermedia* and *P. nigrescens* and then are degraded by cell-associated proteases.

In conclusion, evidence for the binding of *P. gingivalis* fimbriae to lactoferrin and the structures involved in the interaction has been presented.

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