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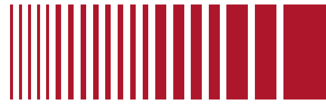

LACTOFERRIN RESEARCH

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Human lactoferrin binding to *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella melaninogenica*

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Human isolates of *Porphyromonas gingivalis* ($n = 16$), *Prevotella intermedia* $n = 82$) and *Prevotella melaninogenica* ($n = 18$) from diseased periodontal pockets were examined for interaction with human lactoferrin (HLf) in a standardized ^{125}I-labeled protein binding assay. The highest HLf binding was found in *P. intermedia* strains, followed by *P. gingivalis* and *P. melaninogenica.* Further characterization of the interaction was performed with 1 representative strain from each species. HLf binding to *P. gingivalis* reached a saturation instantly and was optimal at pH 5.0–6.5. The corresponding values for *P. melaninogenica* were 90 min and pH 3.0–5.5. The HLf binding to the 2 strains seem to be nonspecific. In contrast, *P. intermedia* demonstrated specific binding, and a time-saturability within 60 min with an optimal uptake at pH 6.0–7.5. Scatchard analysis implied 45,000 receptors per cell with an affinity constant of 5.5×10^{-7} M on *P. intermedia* strain 4H. The binding capacity in all 3 strains was affected by the culture medium. HLf binding components in these strains were susceptible to heat or proteases. Binding was eliminated in *P. gingivalis* and was enhanced in *P. intermedia* and *P. melaninogenica* by periodate treatment. Unlabeled HLf or bovine lactoferrin effectively displaced labeled HLf binding. Various proteins and carbohydrates did not inhibit HLf binding. Our data suggest that HLf binds to these periodontitis-associated species and that this mechanism is distinct from the previously known ligand interactions in oral bacteria.

Key words: human lactoferrin; binding; *Porphyromonas gingivalis; Prevotella intermedia; Prevotella melaninogenica;* periodontitis

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Lactoferrin (Lf) is a mammalian iron-binding, acute-phase protein found in saliva, milk and other exocrine secretions (12, 25). Lf concentration in parotid saliva is about 10 μg/ml (12) and its level in the crevicular fluid rises up to ≈ 1.5 mg/ml during localized juvenile periodontitis, gingivitis and adult periodontitis (13).

As a secretory glycoprotein of polymorphonuclear leukocytes (PMN) (27), a role of Lf has been suggested in various host defense processes. Lf mediates the amplification of inflammatory response (34, 48), phagocytosis (1, 10) and regulation of myelopoiesis (7). Inflammatory cells, mainly the PMN, infiltrate the gingival sulcus during the initiation and progression of periodontitis (35, 36). Activated PMN release extracellular antimicrobial substances such as lysozyme, myeloperoxidase (21) and also Lf (25).

Lf is bactericidal during exponential phase and bacteriostatic during the late stationary phase of bacterial growth (3, 5). It may play an important role in host defense at the mucosal surfaces by causing deprivation of iron from bacteria (26, 33). Lf also interacts synergistically with immunoglobulins and causes an increased bacteriostatic effect (38, 47). Lf mediates hydrogen peroxide-dependent and -independent bacterial killing (1). The metal-binding property enables Lf to destabilize the integrity of the outer membrane of gram-negative bacteria and to release lipopolysaccharides (11).

The bactericidal and bacteriostatic properties of Lf on some oral pathogens have been studied (2, 4). However, less is known about the specific binding of this acute-phase protein in oral gram-negative microorganisms commonly found in periodontal lesions. In this study we examined the human Lf (HLf) interaction with black-pigmented oral isolates of *Porphyromonas gingivalis*, formerly *Bacteroides gingivalis* (42), as well as *Prevotella intermedia* and *Prevotella melaninogenica*, formerly *Bacteroides intermedius* and *Bacteroides melaninogenicus* (43). Furthermore, the specific nature of this binding has been elucidated.

Material and methods

Bacteria and culture conditions

A total of 116 strains isolated from diseased periodontal pockets comprising the species *P. gingivalis* ($n = 16$), *P. inter-*

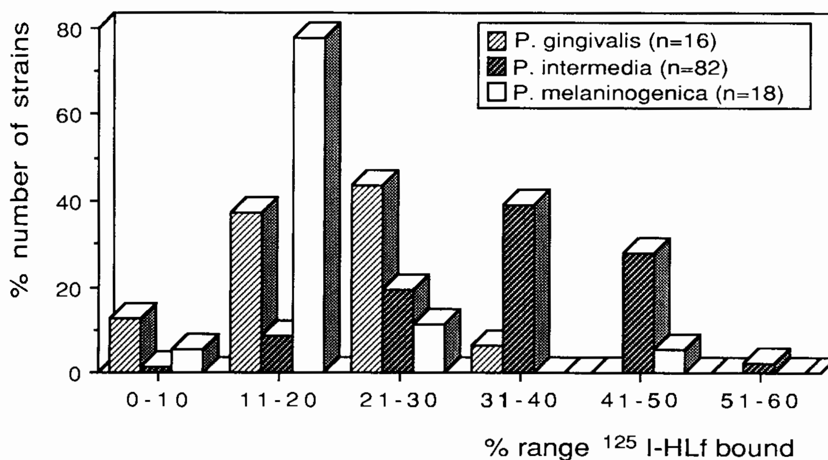

Fig. 1. Frequency distribution (%) of *P. gingivalis*, *P. intermedia* and *P. melaninogenica* strains according to their ^{125}I-HLf binding capacity

media ($n = 82$) and *P. melaninogenica* ($n = 18$) were tested for HLf binding. The respective type cultures 33277 and 25845 from American Type Culture Collection and 9336 from National Collection of Type Cultures were also included in the study. All other strains were isolated from samples routinely examined at our laboratory. Identification of the isolates was based on a previously suggested scheme (17, 20, 45, 46) that includes anaerobic growth, colony and cell morphology, metabolic end products in PYG broth, fermentation of glucose, lactose, cellobiose and esculin, ONPG test, indole production, long-wave UV light fluorescence, and enzyme profiles according to the API-ZYM system (API System, La Balmes les Grottes, Montalieu-Vercieu, France).

For binding experiments, strains were cultured on Brucella agar (BBL Microbiology Systems, Cockeysville, MD) enriched with 5% human erythrocytes, 0.5% laked human erythrocytes and 5 mg/l menadione (Brucella blood agar). To study the influence of culture conditions, strains were also grown on *Bacteroides* medium (44) solidified with 1.8% Bacto agar (BM agar), as well as on BM agar supplemented with either 0.01% sodium bisulfite or 5% human erythrocytes and 0.5% laked human erythrocytes. All plates were incubated at 37°C, under anaerobic conditions (5% CO_2 + 9% H_2 in N_2) for 4–5 d. Bacterial cells were harvested, washed and resuspended in phosphate-buffered saline (PBS), pH 7.2, to a final density of approximately 10^{10} cells/ml. The cell density was determined by counting stained bacterial samples in a Petroff-Hauser chamber.

For characterization of HLf binding, the strains *P. gingivalis* 1145D, *P. intermedia* 4H and *P. melaninogenica* 8FA representing each of the species were selected on the basis of Lf binding. These 3 strains were grown on Brucella blood agar and treated as described above.

Chemicals

HLf (lot 63541) and bovine Lf (BLf; lot 62696) were purchased from US Biochemicals, Cleveland, OH. BLf purified from bovine milk whey was kindly provided by Dr. Hans Burling, Swedish Dairies Association, Malmö, Sweden. Fibronectin was purified from bovine plasma according to Vuento & Vaheri (49). Type-I collagen was obtained from Collagen Corporation, Palo Alto, CA. Immunoglobulin G was obtained from Kabi Vitrum, Stockholm, Sweden. The following chemicals were purchased from Sigma Chemicals, St. Louis, MO: proteins: transferrin, fibrinogen (from bovine plasma), hemin (bovine, type-I), fetuin (from fetal calf serum), and albumin (from bovine serum); carbohydrates: mannose, fucose, ribose, sorbitol, sialic acid type-VIII (from sheep submaxillary glands), N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine; enzymes and enzyme inhibitors: trypsin (type-XIIS), pepsin (from porcine stomach mucosa), proteinase-K (from *Tritirachium album*, type-XIS), trypsin inhibitor (from soybean), phenylmethylsulfonylfluoride and iodoacetamide. All chemicals used for the preparation of buffer solutions were of analytical grade.

^{125}I-Lf binding assay

HLf was labeled according to a modified chloramine-T method (35) with Na(^{125}I), specific activity 629 GBq/mg (DuPont Scandinavia, Stockholm, Sweden), using Iodobeads (Pierce Chemicals, Rockford, IL). Binding assays were performed as described earlier by Naidu et al. (29, 41). In brief, 10^9 bacterial cell in 100 μl PBS were mixed with 100 μl solution of ^{125}I-HLf, radioactivity-adjusted to $2–2.5 \times 10^4$ cpm, i.e. approximately 8 ng protein, in cold PBS. After 1 h incubation at room temperature, 2 ml of ice-cold PBS containing 0.1% Tween 20 was added to the tubes. The suspension was centrifuged at 9500 *g* for 15 min and the supernatant was aspirated. Radioactivity retained in the bacterial pellet was measured in a gamma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Samples were always tested in triplicate and each experiment was repeated at least twice unless otherwise stated.

Displacement and competitive inhibition of ^{125}I-HLf binding

To determine binding specificity, increasing amounts (0.01–300 μg) of unlabeled HLf or BLf in 100 μl PBS was mixed with equal volume PBS containing about 8 ng ^{125}I-HLf. Approximately 10^9 cells of the test strain were added to this solution (final volume 300 μl) and the mixture was incubated at room temperature for the appropriate binding saturation time. Furthermore, increasing amounts (0.01–40 μg) of labeled

Table 1. Influence of culture media on ^{125}I-HLf binding to bacteria

Culture media	Mean ^{125}I-HLf bound (nmol × 10^6) ± SD *P. gingivalis* strain 1145D	*P. intermedia* strain 4H	*P. melaninogenica* strain 8FA
Brucella-blood agar	28±2	41±2	20±1
BM agar	25±1	17±0	9±0
BM+erythrocytes	17±3	51±2	14±0
BM+$NaHSO_3$	17±1	17±0	12±0

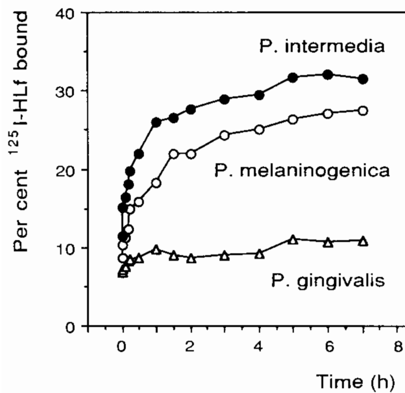

Fig. 2. Time course of ^{125}I-HLf binding to *P. gingivalis* 1145D, *P. intermedia* 4H, and *P. melaninogenica* 8FA

HLf were allowed to interact with 10^9 bacterial cells, either in the absence (=total binding) or in the presence (=nonspecific binding) of a 50-fold excess (calculated using the above binding displacement data) of unlabeled HLf.

The competitive inhibitory effect of various proteins, such as fibronectin, transferrin, fibrinogen, serum albumin, hemin and fetuin, and of various carbohydrates such as D(+)mannose, L(−)-fucose, D(−)ribose, D(−)sorbitol, sialic acid type-VIII, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine on ^{125}I-HLf binding to bacteria was tested. A volume of 100 μl of 0.1% protein or carbohydrate solution was mixed with approximately 10^9 cells of the test strain (100 μl volume) and kept at room temperature for 30 min. Finally, 100 μl ($\approx$8 ng) of ^{125}I-HLf was added and the mixture was incubated at the binding saturation time. ^{125}I-labeled protein binding measurements were made as described.

Enzyme treatment of bacteria

Bacteria were treated with proteases in a total volume of 0.5 ml at room temperature. Trypsin (2500 units/10^9 cells) hydrolysis was performed in 0.15 M PBS, pH 7.4, and the reaction was blocked with soybean trypsin inhibitor. Pepsin (1000 units/10^9 cells) treatment was performed in 0.1 M sodium acetate buffer, pH 4.5, and the hydrolysis was stopped by increasing the pH of the reaction mixture to 7.4. Proteinase K (10 units/10^9 cells) digestion was carried out in 40 mM potassium phosphate buffer, pH 7.5, and the reaction was inhibited by the addition of 0.5 M phenylmethylsulfonylfluoride. All suspensions were incubated under gentle rotation to prevent bacterial sedimentation, for 1 h. The cells were washed thoroughly, resuspended in 0.1 ml PBS and assayed for ^{125}I-HLf binding as described above. Untreated cells suspended in appropriate buffers served as controls for the calculation of percentage susceptibility values.

Heat and periodate treatments of bacteria

One ml of bacterial suspension (10^{10} cells) was heated in a water bath at 50°C and 80°C, under gentle agitation, for 1 h. Furthermore, 1 ml of bacterial suspension (10^{10} cells) was mixed with 5 mg of sodium periodate and the mixture was kept in dark, at 4°C, under gentle shaking, for 24 h. The mixture was then centrifuged at 150 *g* for 5 min to remove the periodate crystals and bacterial cells in the supernatant were aspirated and dialyzed against PBS for 24 h. Finally, the density of the periodate-free cell suspension was adjusted to 10^{10} cells/ml. Cells obtained from the above treatments were tested for ^{125}I-HLf uptake as described.

Results

Strains of *P. gingivalis*, *P. intermedia* and *P. melaninogenica* grown on Brucella blood agar demonstrated differences in ^{125} I-HLf binding. According to the frequency distribution pattern, a majority of strains belonging to *P. gingivalis* were in the 11–30%, *P. intermedia* in the 31–50% and *P. melaninogenica* in the 11–20% HLf binding range (Fig. 1). Thus, *P. intermedia* showed highest HLf binding (mean value 3.8×10^{-7} nmol/10^9 bacteria). The corresponding values for *P. gingivalis* and *P. melaninogenica* were 2.0×10^{-7} and 1.7×10^{-7} nmol/10^9 bacteria, respectively.

The HLf binding capacity of bacteria was altered by different growth conditions (Table 1). Brucella blood agar was optimal for expression of HLf binding in bacteria from all 3 species. Cells of *P. gingivalis* and *P. melaninogenica* grown on BM agar alone, or supplemented with erythrocytes or sodium bisulfite demonstrated a lower HLf binding. However, *P. intermedia* showed an increased binding when grown on BM agar supplemented with erythrocytes.

The further characterization of HLf binding performed with a representative strain from each species grown on Brucella blood agar is described below.

P. gingivalis 1145D demonstrated a binding of 1.6×10^{-7} nmol HLf/10^9 cells. Binding saturation was reached instantly (Fig. 2) and this binding was optimal in the pH range 5.0–6.5. Unlabeled HLf or BLf displaced the ^{125}I-HLf uptake in a dose-dependent manner and required $\approx$250 μg and $\approx$180 μg, respectively, to elicit 50% displacement (Fig. 3). When the binding was performed with increasing ^{125}I-HLf in the presence of a 50-fold excess of unlabeled ligand, the binding to *P. gingivalis* seemed to be nonspecific (Fig. 4). The HLf binding to the bacteria was susceptible to pepsin, resistant to trypsin, proteinase K or sodium periodate (Table 2). Heating the cells at 80 C for 1 h eliminated 53% of the HLf binding (Table 2).

P. intermedia 4H bound 4.4×10^{-7} nmol HLf/10^9 cells. The binding reached a saturation within 60 min (Fig. 2) and was optimal in the pH 6.0–7.5 range. Unlabeled HLf or BLf displaced ^{125}I-HLf uptake in a dose-dependent manner and required $\approx$3 μg of each

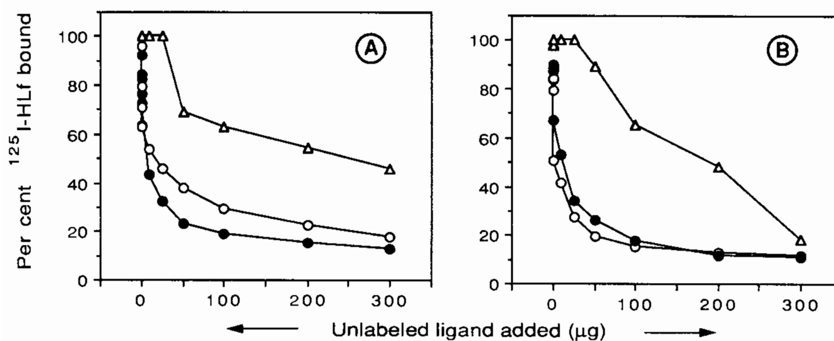

Fig. 3. Dose-dependent displacement of ^{125}I-HLf binding in *P. gingivalis* 1145D (△), *P. intermedia* 4H (●), and *P. melaninogenica* 8FA (○) by unlabeled HLf (A) or unlabeled BLf (B)

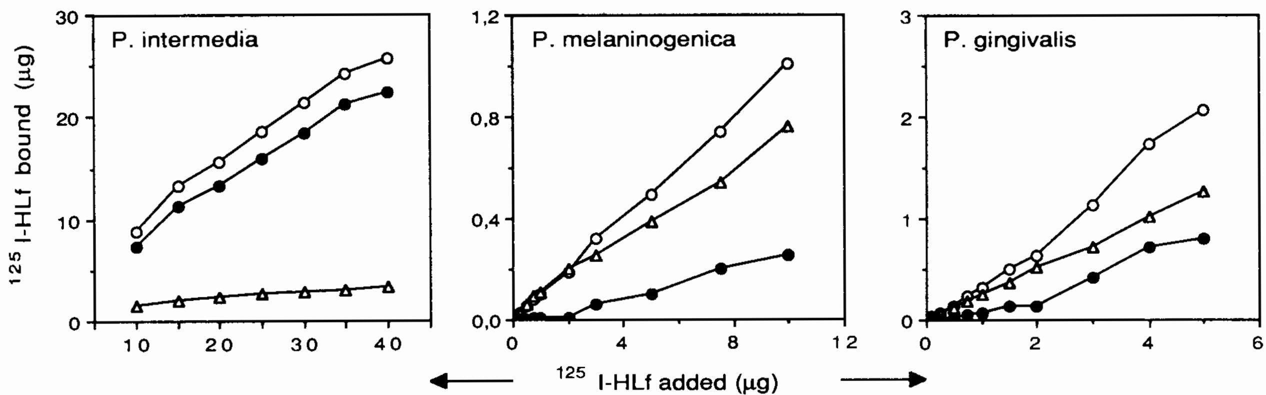

Fig. 4. ^{125}I-HLf binding to *P. gingivalis* 1145D, *P. intermedia* 4H, and *P. melaninogenica* 8FA at increasing concentration of labeled HLf in the presence (nonspecific binding, △) or absence (total binding, ○) of 50-fold excess unlabeled ligand. The specific binding (●) was calculated by substracting nonspecific from total binding values.

protein to elicit a 50% displacement (Fig. 3). The interaction was specific (Fig. 4) with an affinity constant (K_a) of 5.5×10^{-7} M. Scatchard analysis gave an estimation of 45,000 binding sites/bacterium (Fig. 5). Treatment of bacteria with pepsin, trypsin, or proteinase K eliminated HLf binding by 52%, 34% and 26% respectively (Table 2). Periodate treatment enhanced the binding by 18% and heating of the cells at 80°C for 1 h decreased 61% of the HLf binding (Table 2).

P. melaninogenica 8FA demonstrated a binding of 2.8×10^7 nmol HLf/10^9 cells. The binding reached a saturation within 90 min (Fig. 2) and was optimal in the pH range 3.0–5.5. Unlabeled HLf or BLf displaced ^{125}I-HLf uptake in a dose-dependent manner and required ≈4 µg and ≈2 µg respectively, to elicit 50% displacement (Fig. 3). When the binding was performed with increasing ^{125}I-HLf in the presence of 50-fold excess unlabeled ligand, the interaction seemed to be nonspecific (Fig. 4). The HLf binding to the bacteria was susceptible to protease or heat treatment while periodate treatment enhanced the binding by 322% (Table 2).

Cross-displacement or inhibition experiments performed with different proteins or carbohydrates (refer to Material and methods) revealed no interference with the ^{125}I-HLf binding in any of the 3 species tested.

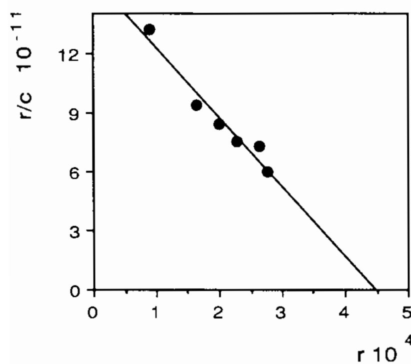

Fig. 5. Scatchard analysis of the specific binding data derived from Fig. 4 for *P. intermedia* 4H, $r/c = nK_a - rK_a$. The molecules of HLf bound to 1 bacterial cell (r) and the molecules of HLf free in the medium (c) were calculated using a MW 76,500 for HLf. The intercept on the *x*-axis represents the number of binding sites per cell (*n*). The slope of the line represents the effective association constant (K_a) in M. Correlation coefficient = 0.97.

Discussion

We demonstrated that strains of *P. gingivalis, P. intermedia* and *P. melaninogenica* interact with HLf in a standardized radioisotope-labeled protein binding assay. ^{125}I-HLf binding in the presence of 50-fold excess unlabeled ligand obtained with 1 strain from each species (Fig. 4) revealed that nonspecific binding accounted for about 10, 75 and 60% of the total binding in *P. intermedia* 4H, *P. melaninogenica* 8FA and *P. gingivalis* 1145D respectively. Assuming that the above data is representative for each species and considering the frequency distribution of the strains according to their binding capacity (Fig. 1), a majority of *P. gingivalis* and *P. melaninogenica* strains can be characterized as low binders (specific binding ≤10%). On the other hand, about 50% of *P. intermedia* strains showed a high binding capacity (specific binding >30%).

Culture conditions have influenced the binding capacity of the strains differently. BM media contain 2% inactivated horse serum and thus are protein-rich. Irrespective of the presence or absence of erythrocytes in the media, the HLf binding was suppressed in *P. gingivalis* and *P. melaninogenica.* On the contrary, under similar conditions, the HLf binding capacity of *P. intermedia* was enhanced. Furthermore, *P. gingivalis* is known to demonstrate strong proteolytic activity (20, 45). Therefore, Lf binding was examined in randomly selected strains from the 3 species in the

Table 2. Enzyme, heat or periodate treatment of bacteria and its influence on ^{125}I-HLf binding

	% decrease in ^{125}I-HLf binding		
Treatment	*P. gingivalis* strain 1145D	*P. intermedia* strain 4H	*P. melaninogenica* strain 8FA
Proteases			
Pepsin	25	52	50
Trypsin	−3	34	11
Proteinase K	8	26	29
Heat			
50°C for 60 min	−2	1	51
80°C for 60 min	53	61	57
Sodium periodate	0	−18	−322

presence or absence of protease inhibitors, i.e. soybean trypsin inhibitor and phenylmethylsulfonylfluoride. No differences were found in either condition (data not shown), suggesting that the bacterial proteolytic activity does not affect the Lf binding pattern.

Binding inhibition data have suggested that the HLf-bacteria interaction is distinct, since various proteins or carbohydrates caused no interference in any of the species tested. However, all 3 species recognized both human and bovine Lf to a comparable degree. In contrast, other human pathogens such as *Neisseria meningitidis* and *Haemophilus influenzae* have been shown to interact only with HLf (40, 41).

Salivary glycoproteins play an important role in the colonization of bacteria to oral surfaces (14, 16, 23). Glycoprotein-bacteria interaction has been suggested in the clearance of bacteria from the oral cavity by agglutination (22, 29) or by competitive blocking of host binding sites (16). Conversely, the glycoprotein adsorption to enamel surface could favor bacterial adherence (15, 23). Salivary compounds such as fibronectin, fibrinogen and proline-rich proteins have been implied in this function and specific binding of these molecules to certain oral bacteria has been demonstrated (14, 15, 18, 22, 23, 28). The interaction of HLf with periodontal pathogens probably acts similarly. Lf has recently been shown to inhibit the adsorption of *Streptococcus mutans* to hydroxyapatite (50).

HLf interaction with *P. intermedia* 4H seems to be mediated by specific receptors. The binding was saturable and readily displaceable. The Scatchard analysis implied approximately 45,000 HLf binding sites per cell with an affinity constant (K_a) of 5.5×10^{-7} M. This receptor density was higher than in *Staphylococcus aureus*, coagulase-negative staphylococci and *Escherichia coli*, but the K_a value is within a comparable range (30–32).

The heat or protease treatment results suggest a proteinaceous nature of the bacterial cell surface components involved in HLf binding. Data from the carbohydrate inhibition experiments have ruled out the involvement of glycosyl residues of HLf in the interaction. In contrast, the HLf binding to eucaryotic cells such as monocytes, hepatocytes and intestinal brush border epithelia is inhibited by fucose or mannose (6, 9, 19, 37). On the other hand, increased HLf binding in *P. melaninogenica* and *P. intermedia* after periodate treatment is probably caused by the elimination of steric hindrance caused by carbohydrate structures on the cell surface or the exposure of additional binding sites, i.e. cryptotopes.

In conclusion, evidence of HLf interaction with *P. intermedia, P. melaninogenica* and *P. gingivalis*, species associated with periodontal diseases, has been presented. The antimicrobial effect of Lf is related to its adsorption to the microbial surface (8). In such interactions, the metal-chelating property of Lf enables damage to lipid bilayers in the outer cell membrane and the release of lipopolysaccharides (11). Whether HLf interaction, specific or non-specific, can elicit similar antimicrobial effect on the periodontitis-associated species remains to be elucidated.

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Effect of lactoferrin on interaction of *Prevotella intermedia* with plasma and subepithelial matrix proteins

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A lactoferrin-binding protein with an estimated molecular mass of 57 kDa was identified in the cell envelope of *Prevotella intermedia* by gel electrophoresis and Western-blot analysis. Peroxidase-labeled bovine lactoferrin and human lactoferrin showed similar specific binding to this protein. Whole cells of *P. intermedia* were also examined for interactions with 5 ^{125}I-labeled plasma and subepithelial matrix proteins. A high degree of binding was found with fibronectin, collagen type I and type IV and laminin, whereas a moderate interaction was detected with fibrinogen. The ability of bovine lactoferrin to affect the interactions of the above proteins with *P. intermedia* was examined. In the presence of unlabeled bovine lactoferrin, a dose-dependent inhibition of binding was observed with all 5 proteins tested. Unlabeled bovine lactoferrin also dissociated the bacterial complexes with these proteins. The complexes with laminin or collagen type I were more effectively dissociated than fibronectin or fibrinogen, whereas the interaction with collagen type IV was affected to a lesser extent. A strain-dependent variation in the effect of bovine lactoferrin was observed. These data establish the presence of a specific lactoferrin-binding protein in the cell envelope of *P. intermedia*. The ability of lactoferrin to inhibit the binding of some plasma and subepithelial matrix proteins to *P. intermedia* could be a protective mechanism against the establishment of this pathogen in the periodontal pocket.

Key words: lactoferrin; fibronectin; fibrinogen; collagen; laminin; binding; inhibition; *Prevotella intermedia*

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Certain bacteria associated with periodontal diseases, such as *Actinobacillus actinomycetemcomitans, Fusobacterium* spp., *Porphyromonas gingivalis* and *Prevotella intermedia,* bind to various plasma and subepithelial matrix proteins, including fibronectin, fibrinogen, collagen and laminin (15, 20, 27, 36). Such interactions are suggested to mediate the adherence of microbes to periodontal tissues, a prerequisite for the establishment of pathogens in the periodontal pocket (5, 11), and may contribute to their virulence.

Beside the constant presence of microorganisms, the immunologic response to the microbial insult and the inflammatory nature of the disease are also important factors that may contribute to the progression of periodontitis (29). The chemical composition of the environment in the periodontal pocket changes during an inflammatory response due to increased exudation and also elevation in the concentration of several humoral defense factors (6).

Lactoferrin is an iron-binding glycoprotein present in saliva and other exocrine secretions (23). Lactoferrin levels in the crevicular fluid of inflamed periodontal pockets rise significantly as a consequence of the neutrophil degranulation (1, 9). Lactoferrin exerts bacteriostatic and bactericidal effects against various microbial pathogens (2–4, 7, 16, 26, 28). This protein was also shown to inhibit adhesion of *Streptococcus mutans* to hydroxyapatite (34) and *Shigella flexneri* to epithelial cells (13).

Recent studies have demonstrated a specific binding of lactoferrin to *P. intermedia* (14, 18) and also the ability of lactoferrin to selectively inhibit the binding of the matrix protein laminin to this bacterium (15). Since knowledge on the specific binding of lactoferrin with *P. intermedia* and the implications of this interaction is limited, this study was therefore conducted to identify the binding components in this bacterium

and to examine the effect of this binding on the interactions of *P. intermedia* with plasma and subepithelial matrix proteins that may mediate adherence of this bacterium to the periodontal tissues.

Material and methods

Bacterial strains

A total of 14 *P. intermedia* strains isolated from diseased periodontal pockets were examined. The strains were previously identified by standard procedures (31, 32) and also tested for lactoferrin binding in a previous study (14). Bacteria were grown on Brucella agar (BBL Microbiology Systems, Cockeysville, MD) enriched with 5% human erythrocytes, 0.5% hemolyzed human erythrocytes and 5 mg/l menadione, at 37 C, under anaerobic conditions (5% CO_2+9% H_2 in N_2), for 3–4 days. Bacterial cells were harvested, washed and resuspended in phosphate-buffered saline, pH 7.2, to a final density of approximately 10^{10} cells/ml. The cell density was determined by counting stained bacterial samples in a Petroff-Hauser chamber. The bacterial suspensions were kept at −80° C until used.

Demonstration of the lactoferrin-binding protein

Whole-cell extracts were prepared by suspending bacteria in electrophoresis buffer containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The suspension was either boiled for 5 min (heated cell extract) or kept at room temperature for 30 min (unheated cell extract). The insoluble cell fragments were removed by centrifugation (15,000 × *g*, 10 min).

Cell envelopes were prepared from *P. intermedia* OMGS105. An 8-ml volume of bacterial suspension (2×10^{10} cells/ml) in Tris-buffered saline, pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride was sonicated (3 × 1 min, 4°C), and the unbroken cells and large debris were removed by centrifugation (4500 × *g*, 15 min). The supernatant was ultracentrifuged at 100,000 × *g* for 60 min. The cell envelope-containing pellet was washed twice and resuspended in Tris-buffered saline, mixed with an equal volume of glycerol and stored at −20°C until used.

Bromphenol blue (0.03%) and sucrose (20%) were added to all samples and SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a linear 4–20% acrylamide gradient slab (130 × 180 mm), using a Protean® II apparatus (Bio-Rad Laboratories, Richmond, CA). The electrophoresis buffer, pH 8.0, contained 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA and 0.2% SDS. The gel slab was pre-conditioned at a constant voltage of 70 V for 2 h. Electrophoresis was run at 300 V for 10 min and then at 150 V until the bromphenol blue dye migrated off the bottom of the slab. The high- and low-molecular-weight standards (Pharmacia, Uppsala, Sweden) were used according to the manufacturer's instructions. All samples were run in quadruplicates in SDS-PAGE in the same slab. The gel was cut into two parts and one part was fixed in a solution containing 11.5% trichloroacetic acid and 3.45% sulfosalicylic acid, for 30 min, and stained with 0.12% Coomassie brilliant blue R-250, 25% ethanol and 8% acetic acid in water, for 4 h. The gel was destained with several changes of a 25% ethanol and 8% acetic acid solution until the background was clear. Proteins in the second part of the non-fixed gel were transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany) at 0.8 mA/cm^2 for 18 h, using a Trans-Blot® cell (Bio-Rad Laboratories). The buffer was 0.025 M Tris, pH 8.3, containing 0.2 M glycin and 20% methanol. The membrane was incubated with 1% Tween 20 in Tris-buffered saline, pH 7.4, for 15 min, at room temperature, to block the free sites on the membrane. Western blotting was performed as previously described (17). In brief, horseradish peroxidase-coupled human or bovine lactoferrin (18) was used at a concentration of ~0.5 μg/ml to probe the membrane at room temperature for 1 h. The membane was thoroughly washed, and a color reaction was developed in 0.1 M sodium acetate buffer, pH 5.0, containing 0.25 mg/ml diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.003% H_2O_2. The reaction was terminated with 5% sodium pyrosulfite.

Isotope-labeled protein binding assay

Human lactoferrin from milk (lot 63541) and bovine lactoferrin from colostrum (lot 69696) were purchased from US Biochemicals, Cleveland, OH. Fibronectin isolated from human plasma according to the method of Vuento & Vaheri (35) was kindly provided by J. Erdei. Fibrinogen from human plasma (lot 77F-9464, Sigma Chemical Co.), collagen type-I (Collagen Corporation, Palo Alto, CA) as well as collagen type-IV (lot 89-0575) and laminin (lot 90-0294), both purified from mouse EHS sarcoma (Collaborative Research, Bradford, MA), were used. All proteins were labeled with Na(^{125}I), specific activity 0.88 GBq/mg (DuPont Scandinavia, Stockholm, Sweden), using Iodo-beads® (Pierce Chemicals Co., Rockford, IL) (22). Binding assays were performed as described earlier by Naidu et al. (24). In brief, 10^9 bacterial cells in 100 μl of phosphate-buffered saline were mixed with a 100-μl solution of ^{125}I-labeled protein with a radioactivity adjusted in phosphate-buffered saline to approximately 20–25 kcpm. After a 1-h incubation at room temperature, 2 ml of ice-cold phosphate-buffered saline containing 0.05% Tween 20 was added to the tubes. The suspension was centrifuged at 4500 × *g* for 15 min and the supernatant was carefully aspirated. The radioactivity retained in the bacterial pellet was measured in a gamma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Samples were always tested in triplicate, and each experiment was repeated at least twice unless stated otherwise. The binding is expressed as the percentage bound of the total labeled protein added.

Inhibition and dissociation of protein binding

Different concentrations (range 0.002–3.3 mg/ml) of unlabeled bovine lactoferrin were examined for competitive binding of ^{125}I-labeled fibronectin, fibrinogen, collagen type I, collagen type IV and laminin to *P. intermedia* OMGS105. For this purpose 0.1 ml of isotope-labeled protein (~30 kcpm) in phosphate-buffered saline, pH 7.2, was mixed with 0.1 ml of unlabeled bovine lactoferrin. A volume of 0.1 ml of bacterial suspension (10^9 cells) in phosphate-buffered saline was added and the mixture was incubated at room temperature for 1 h.

The ability of bovine lactoferrin to block the binding of the isotope-labeled protein to all 14 *P. intermedia* strains was examined separately. A volume of 0.1 ml of bacterial suspension was pre-incubated at room temperature with 0.1 ml of unlabeled bovine lactoferrin solution (final concentration of bovine lactoferrin in the mixture 0.3 or 3.3 mg/ml) for 30 min. To this mixture, 0.1 ml

of ^{125}I-labeled protein was added and the incubation was continued for 60 min.

Finally, the ability of bovine lactoferrin to dissociate the plasma and matrix protein-bacteria complex was examined with strain OMGS105. A volume of 0.1 ml ^{125}I-labeled protein was incubated with 0.1 ml bacterial suspension for 60 min. To this mixture was added 0.1 ml of 3.3 mg/ml bovine lactoferrin in phosphate-buffered saline, and the suspension was incubated for an additional 60 min. The reaction was stopped and the cells washed as described above before the radioactivity retained in the cell pellet was measured. Controls with phosphate-buffered saline instead of bovine lactoferrin solution were included to estimate the maximum (corresponding to 100%) binding of the labeled protein to the cells.

Results

The presence of lactoferrin-binding components in the whole-cell extract or cell envelope of *P. intermedia* strain OMGS105 was examined by SDS-PAGE and Western-blot analysis (Fig. 1). A single distinct horseradish peroxidase-labeled lactoferrin reactive band with an estimated molecular mass of ~ 57 kDa was detected. Both the horseradish peroxidase-labeled bovine and human lactoferrin demonstrated similar binding patterns in OMGS105. In none of the controls, that is, blots not exposed to the ligand or blots incubated with unlabeled lactoferrin prior to the addition of the labeled ligand, were bands detected upon development of the enzyme color reaction. The pattern of lactoferrin-binding components in the whole-cell extracts from the other *P. intermedia* strains was similar to OMGS105 (data not shown).

Strain OMGS105 demonstrated a high degree of binding (> 30% of protein added) to human lactoferrin, bovine lactoferrin, fibronectin, collagens type I and type IV and laminin, whereas the interaction with fibrinogen was moderate to low (Table 1). Similar binding patterns were found with the other *P. intermedia* strains (Table 1).

The interaction of ^{125}I-labeled plasma and subepithelial matrix proteins with OMGS105 was further examined in the presence of increasing amounts (2 μg/ml–3.3 mg/ml) of unlabeled bovine lactoferrin. Bovine lactoferrin caused a dose-dependent inhibition of all 5 proteins tested (Fig. 2). The effect was more pronounced for collagen type I and laminin interactions and required 0.1 and 0.4 mg/ml of unlabeled bovine lactoferrin respectively to cause a 50% inhibition of the interactions. The binding of fibronectin, fibrinogen and collagen type IV to OMGS105 was inhibited by 50% in the presence of unlabeled bovine lactoferrin at concentrations of 1.5, 1.2, and 1.6 mg/ml respectively (Fig. 2).

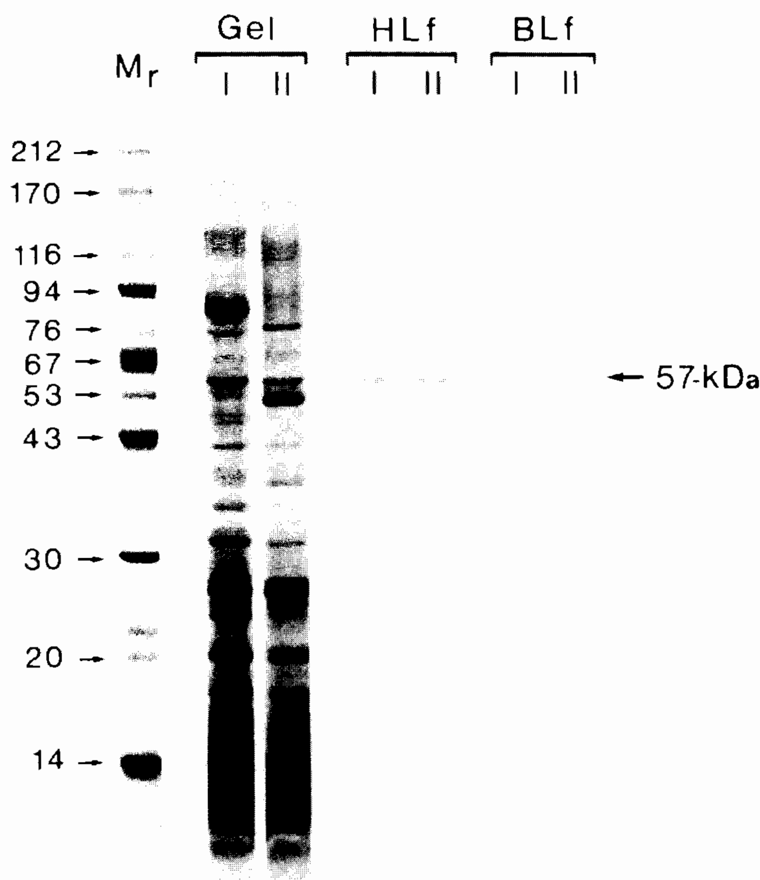

Fig. 1. SDS-PAGE (Gel) and Western blot (HLf and BLf) analyses of unheated (I) and heated (II) whole-cell extracts from *P. intermedia* strain OMGS105. The arrow indicates the position of the protein band (corresponding to an estimated molecular weight of 57 kDa) reactive to horseradish peroxidase-labeled human lactoferrin (HLf) or bovine lactoferrin (BLf) in Western blot.

Table 1. Binding of various ^{125}I-labeled proteins to *P. intermedia*. Mean values ± standard deviation (SD) for 14 strains and values for strain OMGS105.

Protein	Activity (MBq/μg)	Protein added (ng)	% binding Mean ± SD	% binding OMGS105
Human lactoferrin	0.09	8.0	56 ± 6	53
Bovine lactoferrin	0.09	8.0	56 ± 6	52
Fibronectin	0.22	9.5	33 ± 10	35
Fibrinogen	0.18	8.3	17 ± 5	18
Collagen type I	0.24	6.1	41 ± 22	51
Collagen type IV	0.25	5.1	43 ± 4	43
Laminin	0.20	8.3	37 ± 10	38

Bovine lactoferrin blocked the binding of the different ^{125}I-labeled proteins to all 14 *P. intermedia* strains, and the blocking effect depended on the strain and the concentration of bovine lactoferrin in the reaction mixture (Fig. 3). At a concentration of 0.33 mg/ml, bovine lactoferrin inhibited the binding (mean ± standard deviation) of collagen type I (65 ± 28), laminin (53 ± 25), fibronectin (39 ± 20), fibrinogen (35 ± 14)

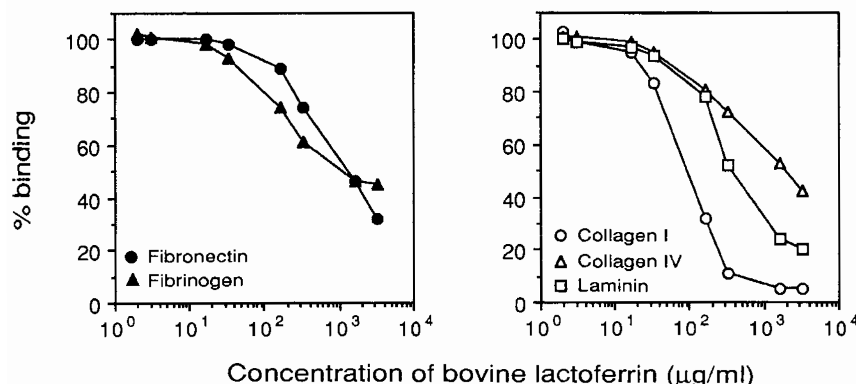

Fig. 2. Relative binding of ^{125}I-labeled fibronectin, fibrinogen, collagen type I, collagen type IV, and laminin to *P. intermedia* strain OMGS105 in the presence of unlabeled bovine lactoferrin. The binding is expressed as a percentage value relative to the control (the bacterial binding of ^{125}I-labeled protein in buffer without unlabeled bovine lactoferrin) that corresponds to 100%.

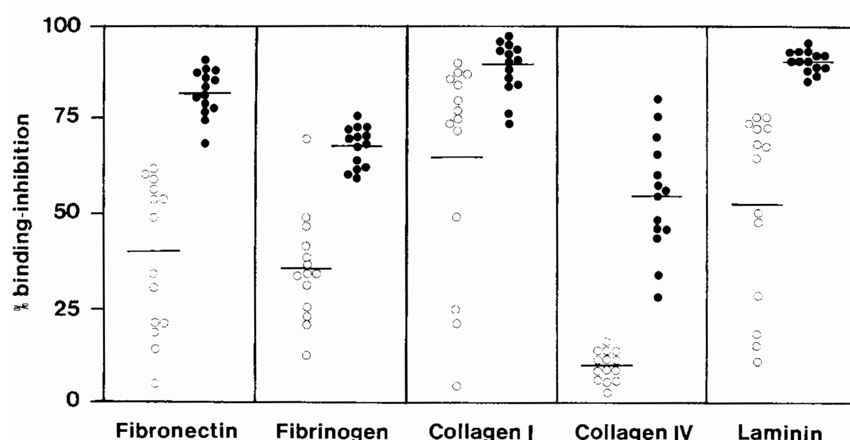

Fig. 3. Inhibition of ^{125}I-labeled plasma and subepithelial matrix protein binding to 14 clinical isolates of *P. intermedia* in the presence of 0.33 mg/ml (open circles) and 3.3 mg/ml (filled circles) unlabeled bovine lactoferrin. The mean values of percentage binding inhibition are indicated as slashes. The inhibition is expressed as a percentage value relative to control (the bacterial binding of ^{125}I-labeled protein in buffer without unlabeled bovine lactoferrin) that corresponds to 0%.

and collagen type IV (10±3) to the strains tested. A 10-fold higher concentration of bovine lactoferrin (3.3 mg/ml) significantly increased the inhibitory effect ($P<0.01$, Student's paired t-test) of all 5 proteins (Fig. 3).

Unlabeled bovine lactoferrin (3.3 mg/ml) effectively dissociated the interactions of ^{125}I-labeled laminin and collagen type-I with strain OMGS105, causing a 52% and 39% dissociation of the interactions respectively (Fig. 4). The interactions of fibronectin and fibrinogen were dissociated to a comparably lesser extent (both <30%), whereas only a weak dissociation (8%) was found of the bacterial complex with collagen type IV (Fig. 4).

Discussion

A specific binding of lactoferrin to *P. intermedia* was reported in an earlier study (14), and the results of Scatchard analysis implied a single class of binding sites with an affinity constant of about 5.5×10^{-7} M. In the present study, we have demonstrated that the lactoferrin-binding sites are proteins with an approximate molecular mass of 57 kDa located in the cell envelope of *P. intermedia* strain OMGS105. A similar lactoferrin-binding protein was also detected in the extracts of 13 other *P. intermedia* strains, thus indicating a common property of this species.

Specific lactoferrin-binding components have earlier been reported in various pathogenic bacteria. Certain members of the family *Enterobacteriaceae*, such as *Escherichia coli*, *Salmonella typhimurium*, *S. flexneri* and *Aeromonas hydrophila*, possess a class of outermembrane proteins, porins, that bind lactoferrin (10, 17, 19, 33). Unlike the lactoferrin-binding protein of *P. intermedia*, porins from these enteric bacteria show a slower electrophoretic mobility when their solutions in SDS are boiled. The lactoferrin-binding proteins of the enteric bacteria and also of *P. intermedia* are recognized by both human lactoferrin and bovine lactoferrin, a property also found in certain *Staphylococcus* spp. (24, 25) but not in *Neisseria gonorrhoeae* and *Neisseria meningitidis* that possess proteins specifically reacting with only human lactoferrin (21, 30).

Viewed from the point of host defense, the lactoferrin interaction with porins of some enteric bacteria may affect the barrier function and alter the outer membrane permeability, with a concomitant increase in the bacterial susceptibility to antibiotics (8, 19). Another defense mechanism suggested for lactoferrin is its ability to inhibit adhesion of *Streptococcus mutans* to hydroxyapatite and possibly affect deposition of this bacterium on the tooth surfaces (34). In addition, fucose-containing peptides from lactoferrin are also known to inhibit the adherence of *S. flexneri* to colon epithelium and probably influence the colonization of this enteropathogen in the intestinal tract (13). The present results show an inhibitory effect of lactoferrin on the interaction of *P. intermedia* with certain

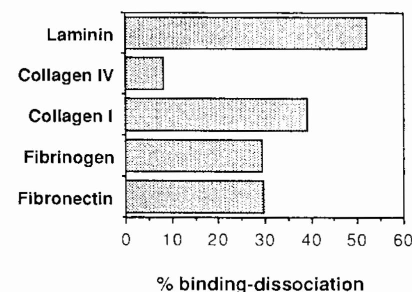

Fig. 4. Dissociation of the binding between ^{125}I-labeled plasma and subepithelial matrix proteins and cells of *P. intermedia* OMGS105 by unlabeled bovine lactoferrin (3.3 mg/ml). The dissociation is expressed as a percentage value relative to control (the binding in the absence of unlabeled bovine lactoferrin) that corresponds to 0%.

plasma and subepithelial matrix proteins. This inhibition was dose-, protein- and strain-dependent. The lactoferrin concentrations required to elicit a 50% displacement of the different proteins were about 100–1000 times higher than the corresponding concentrations found to displace the specific interaction of lactoferrin with *P. intermedia* (14). Lactoferrin exerted both competitive and displacement inhibition, and at a high lactoferrin concentration (3.33 mg/ml), the inter-strain variation was largely diminished with all proteins but collagen type IV. The interaction of collagen type IV with *P. intermedia* showed a higher resistance to the inhibitory effect of lactoferrin and required approximately a 10-fold higher lactoferrin concentration to elicit an inhibitory effect comparable to that of the other proteins tested. A possible explanation for the inhibitory effect could be that the different proteins share a common binding site in *P. intermedia*, probably the 57-kDa cell envelope protein. Similar results were recently reported for *Treponema denticola*, which has a 53-kDa surface protein-mediating binding of the proteins fibrinogen, fibronectin, collagen type I and laminin to this pathogen (12). In whole-cell extract of *P. intermedia*, a laminin-binding protein was previously detected with an estimated molecular weight of 62,000 (15). However, the resolution of proteins from whole-cell extracts of *P. intermedia* at the molecular range of 60 kDa is rather critical, and it is therefore possible that the same protein binds to both laminin and lactoferrin.

In conclusion, lactoferrin binds to a ~57-kDa cell envelope protein of *P. intermedia*. This binding can inhibit the interactions of this bacterium with some plasma and matrix proteins. The lactoferrin-mediated effect could be a protective mechanism against the establishment of *P. intermedia* in the periodontal pocket by inhibiting the bacterial adherence to the subepithelial matrix protein-rich tissues. The latter phenomenon remains to be elucidated.

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