Inhibitory effect of lactoferrin on the adhesion of Prevotella nigrescens ATCC 25261 to hydroxyapatite

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Abstract: Prevotella nigrescens ATCC 25261 (P. nigrescens) cells adhere well to hydroxyapatite treated with citrate (CHA), but the attachment is drastically inhibited by lactoferrin (LF). To determine the nature of the iron-free LF responsible for inhibiting P. nigrescens cell attachment, this study tested the duration and frequency of LF treatment of CHA and the effects of divalent and trivalent ferric ions. The inhibitory effect on the attachment of P. nigrescens was somewhat higher with bovine LF than human LF. Apo LF effectively inhibited P. nigrescens cell attachment to CHA, and almost abolished attachment at a concentration of 0.4 mg/ml. Fe³⁺ saturated LF was unable to inhibit attachment, whereas Fe²⁺ showed a slight effect under the same conditions. The LF adsorbed rapidly to CHA in less than 10 min. With a lower concentration (0.1 mg/ml) of LF, only three treatments of CHA were required for the maximum inhibition of P. nigrescens cell attachment. The quantity of LF adsorbed to the hydroxyapatite (HA) and to P. nigrescens cells was determined by use of [³H]-LF. Approximately 25 µg of LF protein adsorbed to 5 mg of HA at saturation, and approximately 0.25 µg of LF did so to the 6 x 10⁶ cells. (J. Oral Sci. 42, 125-131, 2000)

Key words: lactoferrin; Prevotella nigrescens; adhesion; inhibition; hydroxyapatite.

Introduction

Oral gram-negative anaerobic rods such as Actinobacillus actinomycetemcomitans, Fusobacterium spp., Porphyromonas gingivalis and Prevotella (P.) intermedia are thought to be associated with periodontal diseases. The primary habitat of these bacteria has been assumed to be the periodontal pocket (1). These organisms can bind to various connective tissue proteins such as fibronectin, fibrinogen, collagen and laminin (2-5). It has been suggested that such interactions between bacterial cell surface components and proteins mediate the adherence of microbes to periodontal tissues, prior to establishment in the periodontal pocket (6).

P. intermedia/P. nigrescens isolates are commonly found in the oral cavity of both periodontally healthy and diseased individuals as well as those with various extraoral infections (7-9). The group consists of two species, P. intermedia and P. nigrescens, that are indistinguishable by routine phenotypic methods (10-12). P. intermedia has been associated with periodontal disease, whereas P. nigrescens has been recovered from both periodontally healthy and diseased individuals (13-15).

At sites of microbial invasion, early inflammatory reactions lead to increased concentrations of certain antimicrobial substances released from infiltrating leukocytes (16,17). Lactoferrin (LF), a glycoprotein present in many external secretions (18), is also a constituent of the specific granules of polymorphonuclear leucocytes. The LF excreted by these cells is found in increased concentrations in the gingival crevicular fluid of the diseased periodontium (19, 20). As one of the nonspecific host defense proteins, LF has both bacteriostatic and bactericidal effects on a variety of microbial pathogens (21, 22). Recently, LF has been shown to inhibit the binding of P. intermedia to fibrinogen, fibronecint, laminin and collagen (23). Based on these findings, it has been suggested that LF performs a role in the prevention of disease development, possibly through the elimination of these bacteria from periodontal soft tissues.
More recently, we have shown that *P. nigrescens* exhibits remarkable binding to hydroxyapatite (HA) treated with citrate (CHA)(24). This finding indicates that *P. nigrescens* cells can become attached to a non-desquamating root surface in the periodontal pocket. The adsorption of citrate to HA has also been tested and the amounts of adsorbed citrate to 5 mg of HA is estimated at approximately 360 nmole, as measured by fluorometric microanalysis (25).

The purpose of the present study was to clarify the role of LF in the adhesion of *P. nigrescens* cells to CHA used to mimic a root surface in the periodontal pocket.

**Materials and Methods**

**Bacterial strain and culture conditions**

*P. nigrescens* was obtained from the culture collection of our laboratory. Stock cultures were stored in 1 % skim milk at -80°C until used. The *P. nigrescens* strain was preincubated in GAM broth (Nissui, Japan) in an anaerobic jar for 24 h at 37°C in an atmosphere of 95 % N₂ and 5 % CO₂. The fresh bacterial cells were then inoculated into GAM broth supplemented with 740 kBq of [³H]-thymidine (ICN Biochemicals, CA, USA) per ml. Bacteria used in the adhesion assay were grown to the early stationary phase at 37°C under an anaerobic condition (BBL GasPak Anaerobic System, Becton Dickinson Microbiology Systems, MD, USA). Bacterial cells were harvested by centrifugation and washed twice with buffered KCl (0.05 M KCl containing 1 mM KH₂PO₄, 1 mM KH₂PO₄, 1 mM CaCl₂ and 0.1 mM MgCl₂ at pH 6.2). The washed cells were then suspended into GAM broth supplemented with 740 kBq of [³H]-thymidine (ICN Biochemicals, CA, USA) per ml. Bacteria used in the adhesion assay were grown to the early stationary phase at 37°C under an anaerobic condition (BBL GasPak Anaerobic System, Becton Dickinson Microbiology Systems, MD, USA). Bacterial cells were harvested by centrifugation and washed twice with buffered KCl (0.05 M KCl containing 1 mM KH₂PO₄, 1 mM KH₂PO₄, 1 mM CaCl₂ and 0.1 mM MgCl₂ at pH 6.2). The washed cells were then suspended in BSA-KCl; buffered KCl supplemented with 5 mg per ml of bovine serum albumin (Sigma Chemical Co., MO, USA). The suspensions were adjusted to a level of 6 × 10⁷ bacteria per ml based on a standard curve relating optical density (550 nm) to the number of bacterial cells, as determined by microscopic counting.

**Bacterial adhesion assays**

Bacterial adhesion to HA was studied with the use of citrate- (trisodium citrate dihydrate: Wako Pure Chemical Industries, Ltd., Japan) treated HA beads (Nihon Chemical Co., Japan). Before the assay, 5 mg of the beads were equilibrated overnight in buffered KCl at room temperature (RT). After treatment of HA with BSA-KCl for 30 min at RT to block any uncoated bead surfaces (26), the beads were then washed twice with buffered KCl and incubated with an adequate concentration of citrate solution for 15 min at RT. The beads were again washed and the liquid was removed, then the CHA beads were incubated with [³H]-labeled bacterial cells, using an adequate number of bacterial cells in 125 μl of BSA-KCl. After one hour of continuous rotation at RT, the beads were washed twice with buffered KCl, and transferred to scintillation vials. The number of cells that had become attached was determined by direct scintillation counting (LSC-5200: Aloka, Japan). The influence of the presence of LF on *P. nigrescens* attachment to CHA was also studied by mixing with *P. nigrescens* cells.

All assays were conducted in duplicate, and most experiments were performed at least 5 times.

**Adsorption of [³H]-LF to HA beads or *P. nigrescens* cells**

To determine the quantity of bovine LF which adsorbed to the HA beads and *P. nigrescens* cells, 5 mg samples each of HA beads and *P. nigrescens* cells (6 × 10⁷) were incubated with 125 μl of various concentrations of LF, which had been radiolabeled with [³H]-formaldehyde as described by Jentoft and Dearborn (27). The [³H]-LF was diluted with unlabeled LF solution so as to contain 2,500 dpm per μg protein. After 1 hr of incubation at RT, the mixture was then washed twice with buffered KCl and transferred to scintillation vials for counting.

**Statistical analysis**

Comparisons between each data were made with Mann-Whitney U test.

**Results**

The influence of the presence of LF on the adhesion of *P. nigrescens* cells to CHA

The attachment of *P. nigrescens* to CHA treated with increasing amounts of LF, ranging from 0 to 0.4 mg/ml was tested. In the absence of LF, *P. nigrescens* cells were attached dose-dependently to CHA up to a citrate concentration of 2.0 mM (Fig. 1). In the presence of LF, the attachment of cells was remarkably lower. Attachment was almost completely inhibited at a LF concentration of 0.4 mg/ml (Table 1).

The influence of the treatment of CHA with LF on the attachment of *P. nigrescens*

The number of bacteria binding to LF-treated CHA fell dramatically compared with non-treated CHA. At a concentration of 0.4 mg/ml, LF almost completely abolished the citrate-mediated attachment of *P. nigrescens* (Table 2).

Influence of the treatment of *P. nigrescens* cell with LF on the attachment to CHA

The number of bacteria binding to CHA was not affected by the treatment of *P. nigrescens* cell with LF (Table 3).
Comparison of the inhibitory effect between bovine LF and human LF

Bovine LF-treated and human LF-treated CHA were tested. The inhibitory effect on *P. nigrescens* attachment to CHA was much higher with bovine LF than with the human LF used. The inhibitory rate was well over 4 times higher with bovine LF than human LF (Table 4).

Effect of the duration of LF-treatment of CHA on the attachment of *P. nigrescens*

The cell count was almost the same for bacteria binding to CHA for various durations (5, 10, 15, 20 and 25 min) (Table 5). It was, therefore, concluded that 15 min of LF-treatment of CHA was sufficient to produce the maximum inhibitory effect.

Effect of frequency of LF-treatment of CHA on the attachment of *P. nigrescens*

The number of bacteria binding to CHA treated with LF decreased in proportion to the frequency of treatment. Even when the concentration of LF was relatively low (0.1 mg/ml, 3 times), *P. nigrescens* was unable to attach itself to the CHA (Table 6).

Effect of iron-treatment of CHA treated with LF on the attachment of *P. nigrescens*

Citrate- (2.0 mM, 125 µl for 15 min) treated HA (5 mg) was treated successively with LF (0.4 mg/ml, 125 µl for...
Table 5 Effect of duration of LF-treatment of CHA on the attachment of \textit{P. nigrescens}

<table>
<thead>
<tr>
<th>duration (min)</th>
<th>attached bacterial cells$^*$ (×10$^6$ cells/5 mg HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.05 ± 2.14</td>
</tr>
<tr>
<td>5</td>
<td>6.27 ± 0.48</td>
</tr>
<tr>
<td>10</td>
<td>3.57 ± 0.78</td>
</tr>
<tr>
<td>15</td>
<td>2.88 ± 0.35</td>
</tr>
<tr>
<td>20</td>
<td>2.82 ± 0.36</td>
</tr>
<tr>
<td>25</td>
<td>2.90 ± 0.23</td>
</tr>
</tbody>
</table>

$^*$Mean ± SD of 8 assays.  *P<0.01

Table 6 Effect of frequency of LF (conc.: 0.1 mg/ml) treatment of CHA on the attachment of \textit{P. nigrescens}

<table>
<thead>
<tr>
<th>frequency (times)</th>
<th>attached bacterial cells$^*$ (×10$^6$ cells/5 mg HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.05 ± 2.14</td>
</tr>
<tr>
<td>1</td>
<td>44.19 ± 3.36</td>
</tr>
<tr>
<td>2</td>
<td>7.87 ± 1.70</td>
</tr>
<tr>
<td>3</td>
<td>1.65 ± 0.49</td>
</tr>
<tr>
<td>4</td>
<td>1.03 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>1.02 ± 0.10</td>
</tr>
</tbody>
</table>

$^*$Mean ± SD of 8 assays.  *P<0.01

Table 7 Effect of Fe$^{3+}$ or Fe$^{2+}$ on \textit{P. nigrescens} attachment to LF (conc.: 0.4 mg/ml)-treated CHA

<table>
<thead>
<tr>
<th>CHA(+LF): control</th>
<th>attached bacterial cells$^*$ (×10$^6$ cells/5 mg HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_2$(1 mM)</td>
<td>0.98 ± 0.15</td>
</tr>
<tr>
<td>FeCl$_2$(2 mM)</td>
<td>25.42 ± 3.89</td>
</tr>
<tr>
<td>FeCl$_2$(3 mM)</td>
<td>37.71 ± 2.87 $^{a-1}$</td>
</tr>
<tr>
<td>FeCl$_2$(4 mM)</td>
<td>38.50 ± 3.29 $^{a-2}$</td>
</tr>
<tr>
<td>FeCl$_2$(5 mM)</td>
<td>37.09 ± 6.18 $^{a-3}$</td>
</tr>
<tr>
<td>FeCl$_3$(1 mM)</td>
<td>1.20 ± 0.09</td>
</tr>
<tr>
<td>FeCl$_3$(2 mM)</td>
<td>4.00 ± 1.05</td>
</tr>
<tr>
<td>FeCl$_3$(3 mM)</td>
<td>9.22 ± 1.88</td>
</tr>
<tr>
<td>FeCl$_3$(4 mM)</td>
<td>21.16 ± 1.84 $^{b-1}$</td>
</tr>
<tr>
<td>FeCl$_3$(5 mM)</td>
<td>21.36 ± 1.65 $^{b-2}$</td>
</tr>
<tr>
<td>FeNH$_4$SO$_4$(1 mM)</td>
<td>2.42 ± 0.18</td>
</tr>
<tr>
<td>FeNH$_4$SO$_4$(2 mM)</td>
<td>15.02 ± 1.15</td>
</tr>
<tr>
<td>FeNH$_4$SO$_4$(3 mM)</td>
<td>31.84 ± 2.84</td>
</tr>
<tr>
<td>FeNH$_4$SO$_4$(4 mM)</td>
<td>38.00 ± 2.99 $^{c-1}$</td>
</tr>
<tr>
<td>FeNH$_4$SO$_4$(5 mM)</td>
<td>39.28 ± 3.17 $^{c-2}$</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$SO$_4$(1 mM)</td>
<td>1.32 ± 0.31</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$SO$_4$(2 mM)</td>
<td>3.12 ± 0.20</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$SO$_4$(3 mM)</td>
<td>7.99 ± 0.92</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$SO$_4$(4 mM)</td>
<td>11.72 ± 1.92 $^{d-1}$</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$SO$_4$(5 mM)</td>
<td>11.58 ± 0.91 $^{d-2}$</td>
</tr>
</tbody>
</table>

$^*$Mean ± SD of 8 assays.  \*a-1: *a-2: *a-3: *b-1: *b-2, *c-1: *c-2 and *d-1: *d-2 show no significance. Others show critical significance (P<0.01).
15 min), and the LF-treated CHA was then treated with various concentrations (1.0-5.0 mM) of ferric (FeCl3·6H2O, FeCl2·4H2O, FeNH4(SO4)2·12H2O and Fe(NH4)2(SO4)2·6H2O) solutions for 15 min. In these experimental conditions, especially with the use of trivalent ferric ion solutions, the number of bacteria attaching to LF-treated CHA recovered drastically. When trivalent ferric ions such as FeCl3·6H2O and FeNH4(SO4)2·12H2O were used, the LF-mediated inhibitory effect was completely restored at concentrations of more than 3 mM and 4 mM, respectively. However, divalent ferric ions such as FeCl2·4H2O and Fe(NH4)2(SO4)2·6H2O had only weak effects on the recovery of P. nigrescens attachment (Table 7).

[^H]-LF binding to bacterial cells

When the binding of[^H]-LF to bacterial cells was studied in a range of concentrations (45-225 μg/125μl), the data showed that P. nigrescens cells had a low affinity for LF (approx. 0.25 μg/6 x 10^8 cells)(Fig. 2).

[^H]-LF binding to HA

The quantity of LF adsorbed to HA was determined using[^H]-bovine LF. The amount of LF that bound to HA increased gradually depending on the amount of added LF. Approximately 25 μg of LF was adsorbed to 5 mg of HA at saturation (Fig. 3).

Discussion

It is well known that the presence of LF in a wide range of human secretions, including saliva, milk, tears, pancreatic fluid, seminal fluid, bile and synovial fluid, plays an important role in host defense mechanisms against bacterial infection (28). It is also known that many anti-microbial salivary proteins, such as lysozyme, lactoperoxidase, LF and secretary IgA, can be adsorb to HA and thereby affect the colonization of oral bacteria (29,30). Since the attachment of oral bacteria to oral hard surfaces is undoubtedly important in the etiology and progress of oral disease, clarification of the nature of the LF interaction with HA would facilitate modulation of the adsorption of P. nigrescens to tooth root surfaces in the periodontal pocket.

It has previously been demonstrated that P. nigrescens has the ability to bind to LF. The strain P. nigrescens ATCC 25261 used in this report showed relatively lower affinity for LF than did HA, but the same adsorbing range was observed as the data of de Lillo et al. (31,32).

LF of both bovine and human origin inhibited bacterial adhesion, but the bovine protein exerted a slightly greater inhibitory effect compared to the human version. Bovine LF and human LF have slight differences in their molecular weight and S-value, and bovine LF might have shown a somewhat higher inhibitory rate. The present data show that CHA is able to adsorb LF up to 25 μg per 5 mg of HA and this adsorption drastically reduces the binding of P. nigrescens cells to HA. The binding is fully recovered by trivalent ferric iron-treatment, so the efficacy of apo-LF (iron-free) might be regarded as specific. By contrast, there was only a weak effect on the adsorption of P. nigrescens when apo-LF-treated CHA was re-treated with divalent ferric iron.

The inhibitory effect of LF on Streptococcus mutans adhesion to HA (33) and E. coli adhesion to, and invasion of, Hela cells (34) has been suggested by virtue of the cationic nature of this glycoprotein. The LF-mediated inhibition of P. nigrescens adsorption to HA might also be considered to result from a variation of the electric charge on the HA surface consequent to the binding of LF. This interaction would, therefore, impede the further binding of P. nigrescens on the negatively charged LF-treated CHA. Another possibility is that LF interferes with the adhesion of P. nigrescens to CHA through blocking of the binding sites.

LF is present in the crevicular fluid, in quantities of 0.6 μg/ml, 1.5 μg/ml, 1.4 μg/ml and 1.7 μg/ml in normal, gingivitis, periodontitis and LJP samples, respectively (35). Similar data has been reported for the elevation of LF levels in the GCF of periodontitis patients (1.0-1.5 mg/ml) compared to healthy subjects (0.5 mg/ml) (35,36). These data suggest that this might represent a useful factor controlling not only the growth of P. nigrescens, but also its adsorption to CHA.

Acknowledgments

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References