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Effects of zinc and copper on adhesion and hemagglutination of *Prevotella intermedia* and *Prevotella nigrescens*

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This study investigated the mechanism of protein attachment to the surface of the putative periodontal pathogens *Prevotella intermedia* and *Prevotella nigrescens* in artificial gingival crevicular fluid, and ways to increase protein attachment to the bacterial cells. The effects of cations on protein attachment, bacterial adhesion, and hemagglutination were examined, and cation-binding components on both bacterial species were identified. The presence of cations, especially zinc, copper and cerium, increased attachment of human serum proteins to both bacterial species. In contrast, the presence of hydrophobic inhibitors or sugars had little effect. Protein attachment was reduced by heat treatment of the bacterial cells. Pretreatment of bacteria with human serum proteins inhibited adhesion of both species to buccal epithelial cells and hemagglutination. These effects were enhanced by the presence of zinc and copper during pretreatment. Using a chelating column, specific zinc- and copper-binding proteins were identified on the surfaces of both bacterial species.

Key words: adhesion; cation; hemagglutination; *Prevotella*; serum

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Bacterial adhesion to oral surfaces is observed during the first stage of dental plaque accumulation and is implicated in dental caries and periodontal disease (4, 6). The onset of periodontal disease may be related to the adhesion and settling of periodontal pathogens in the gingival sulcus, where soft tissue surfaces, such as oral epithelium, and hard tissues, such as teeth, are targets for bacterial adhesion. The gingival sulcus is constantly covered with gingival crevicular fluid (GCF). Bacteria that adsorb to oral surfaces are usually suspended in this fluid before adhesion and are invariably covered with the same molecules as that found in the fluid, especially the proteins. Therefore, it is important to consider how a GCF coating on bacterial cells affects their adhesion and aggregation. Some investigators have reported that the presence of saliva or serum inhibits adhesion and hemagglutination of periodontal pathogens (12). By intentionally increasing the amount of attached protein, rather than periodontal pathogens, it may be possible to inhibit the outbreak or progression of periodontal diseases.

In this study, we sought to clarify the mechanism by which proteins originating in artificial GCF attach to the surfaces of the putative periodontal pathogens *Prevotella intermedia* and *Prevotella nigrescens*. We also aimed to establish a method to increase attachment of human serum proteins to bacterial cells, and to investigate the effect of these proteins on bacterial adhesion and hemagglutination. Further, we sought to study the effects of zinc and copper on protein attachment, bacterial adhesion, and hemagglutination., and identify zinc- or copper-binding components on the surfaces of these bacteria.

Material and methods

Bacterial cells

P. intermedia ATCC25611 and *P. nigrescens* ATCC33563 were used throughout this study. The bacteria were inoculated and grown in Gifu anaerobic medium (GAM) broth (Nissui, Tokyo, Japan) under anaerobic conditions (BBL GasPak anaerobic system, Becton Dickinson Microbiology System, Cockeysville, MD) at 37°C overnight. The cells were harvested in late logarithmic phase by centrifugation at 3000 *g* at 4°C for 15 min, washed twice in phosphate buffered saline (PBS) (5 mM sodium phosphate, 150 mM

sodium chloride; pH 7.2) and suspended in the same buffer. Bacterial cells were quantified using a standard curve relating optical density to the number of bacterial cells determined by microscopy.

Human serum proteins

Human serum proteins (Nippon Bio-test Laboratories Inc., Tokyo, Japan) were heated at 56°C for 60 min to inactivate degradative enzymes, supplemented with sodium azide, filtered (Millipore, Billerica, MA), and dialyzed against PBS to remove inorganic substances. Protein concentration was estimated by the method of Lowry et al. (11), using bovine. serum albumin as the standard.

Radiolabeling of human serum proteins

The labeling of human serum proteins with [^{3}H] or [^{14}C] was performed as described by Jentoft et al. (8). After labeling, protein-containing solutions were dialyzed for 3 days against physiological saline containing 0.04% sodium azide to eliminate unincorporated label. The radioactivity of each solution was measured by direct scintillation counting in a Model 2000 CA scintillation counter (Packard Instrument Co., Inc., Meriden, CT), and the protein concentration was calculated from the radioactivity.

Attachment of human serum proteins to bacterial cells

PBS 1 ml containing 1 mg [^{3}H]-labeled human serum proteins was added to 1×10^9 bacterial cells in polypropylene microcentrifuge tubes (Sarstedt, Rommelsdorf, Germany). The suspension was incubated at room temperature for 60 min and washed twice in PBS. The bacterial cells were transferred to a scintillation vial and counted to calculate the amount of attached proteins, as described above.

To examine the effect of cation-bridge binding on attachment, a 1 mM solution of the chloride of calcium, magnesium, iron, zinc, copper, nickel, manganese, cerium, or aluminum was used. To investigate the effect of hydrophobic interactions, a 1 mM solution of lithium chloride, sodium thiocyanate or 0.1% hexadecane was used. To study the effect of lectin-ligand binding, 1 mM solution of sugar, such as D-glucose, D-mannose, D-fucose, D-galactose, D-lactose, D-galactosamine, N-acetyl-D-galactosamine, D-glucosamine or N-acetyl-D-glucosamine, was used. We also investigated the effect of heating bacteria at 80°C for 5 min on the attachment of proteins. Attachment assays were performed in duplicate, and all experiments were performed four times ($n = 8$). The data were analyzed using the Mann–Whitney *U*-test.

Bacterial adhesion to epithelial cells

We used a modification of the *in vitro* assay described by Gibbons et al. (7). Human epithelial cells were collected from oral mucosal surfaces, suspended in PBS, and washed free of unattached bacteria using Ultrafree®-MC membrane filter tubes (Amicon, Millipore, Billerica, MA) with low-speed centrifugation. The cell suspensions were adjusted to approximately 10^5 cells ml^{-1}. A mixture of 150 µl suspensions of epithelial cells and 150 µl suspensions of bacteria (1×10^9 cells) was incubated at 37°C for 30 min. After incubation, the epithelial cells were washed free of unattached bacteria in membrane filter tubes, as described above. The washed epithelial cells were directly smeared onto glass plates and stained with crystal violet. The number of bacteria adhering to epithelial cells was determined by direct microscopic counting. Data were analyzed by the Mann–Whitney *U*-test ($n = 20$). Adhesion was also determined for the bacterial cells treated with human serum proteins for 60 min and washed in PBS.

Hemagglutination assay

Hemagglutination assays were performed with sheep erythrocytes in round-bottomed microtitration plates. Multiple 50 µl dilutions of bacterial suspensions (maximum optical density, 1.0) were mixed with 50 µl 2% washed sheep erythrocytes in PBS. The plates were incubated at room temperature for 60 min, and the bacterial suspension dilutions that inhibited hemagglutination were determined visually. Hemagglutination was also assessed for the bacterial cells treated with human serum proteins.

SDS-PAGE and fluorography of the attached human serum proteins

Approximately 2×10^9 bacterial cells were incubated with 400 µg [^{14}C]-labeled human serum proteins in 0.5 ml of PBS at 4°C for 60 min in polypropylene microcentrifuge tubes. After three washes in PBS, the bacterial cells were boiled for 5 min in 100 µl 80 mM Tris-HCl buffer (pH 8.64) containing 80 mM boric acid, 10% sucrose, 0.37% EDTA, 10% 2-mercaptoethanol, 6 M urea and 4% sodium dodecyl sulfate, as described by Björck et al. (1). After centrifugation, the supernatants, which contained the labeled attached proteins, were analyzed on 12.5% SDS-polyacrylamide gels according to the method of Laemmli (9). Samples of 40 µl were electrophoresed at a constant current of 30 mA overnight and were prepared for fluorography as described by Bonner et al. (2). Briefly, the gels were equilibrated twice in dimethyl sulfoxide (DMSO) for 60 min and with 2,5-diphenyloxazale-DMSO for 180 min. The gels were washed thoroughly in water and dried in a vacuum on filter paper. Human serum proteins attached to the surface of bacterial cells were identified by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at − 80°C.

Cation-binding components on bacterial cells

The surface components of bacterial cells were collected by incubation in 0.1% 3-[(3-cholamidopropyl) dimethyl-ammonio] propanesulfonic acid (CHAPS; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) at 4°C for 30 min. After centrifugation, the supernatants were filtered, and dialyzed.

Human serum proteins 1 mg or the above samples in binding buffer (20 mM sodium phosphate, 500 mM sodium chloride; pH 7.2) were loaded onto HiTrap™ Chelating HP column (Amersham Pharmacia Biotech, Buckinghamshire, UK) that had previously been treated with a 100 mM solution of the chloride of iron, zinc, copper, nickel, cerium or aluminum. The attached components were eluted with 1 ml elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 50 mM ethylenediaminetetraacetic acid; pH 7.2), and 20 µl samples were loaded onto SDS-polyacrylamide gels. The gels were stained with silver nitrate for analysis.

Results

Attachment of human serum proteins to bacterial cells

The total amounts of human serum proteins attached to *P. intermedia* and *P. nigrescens* were 4.29 and 4.12 µg per 10^9 cells, respectively.

Effects of cations and heating on attachment

The presence of cations, especially zinc, copper, and cerium, increased attachment

of human serum proteins to the surface of both *P. intermedia* and *P. nigrescens* cells (Table 1). Zinc also had a concentration-dependent effect on attachment of proteins.

Heat treatment of both species inhibited the attachment of proteins to bacteria. In contrast, the presence of other substances, such as hydrophobic binding inhibitors or sugars, had little effect on the attachment of proteins to the surface of either bacterial species (data not shown).

Table 1. The effects of cations and heating on attachment of human serum proteins

Substance	*P. intermedia* ATCC25611	*P. nigrescens* ATCC33563
PBS (non cation)	4.29 (0.35)	4.12 (0.27)
$CaCl_2$ 1 mM	5.13 (0.22)[a]	4.75 (0.22)[a]
$MgCl_2$ 1 mM	5.23 (0.44)[a]	4.79 (0.43)[a]
$FeCl_2$ 1 mM	5.20 (0.48)[a]	8.22 (0.66)[a]
$ZnCl_2$ 1 mM	82.81 (10.66)[a]	62.72 (9.61)[a]
$CuCl_2$ 1 mM	57.31 (8.42)[a]	58.16 (9.11)[a]
$NiCl_2$ 1 mM	7.21 (1.44)[a]	5.34 (0.77)[a]
$MnCl_2$ 1 mM	9.49 (1.04)[a]	12.30 (1.13)[a]
$FeCl_3$ 1 mM	11.23 (1.24)[a]	9.49 (1.26)[a]
$CeCl_3$ 1 mM	57.15 (7.13)[a]	56.13 (6.65)[a]
$AlCl_3$ 1 mM	4.55 (0.84)[a]	4.78 (0.59)[a]
$ZnCl_2$		
0.1 mM	11.63 (2.89)[a]	11.58 (1. 01)[a]
0.5 mM	41.12 (7.66)[a]	36.18 (5.68)[a]
1 mM	82.81 (10.66)[a]	62.72 (9.61)[a]
2 mM	105.12 (22.45)[a]	87.87 (15.89)[a]
5 mM	107.87 (15.89)[a]	82.81 (10.66)[a]
Heat treatment of bacteria	2.58 (0.22)[a]	2.85 (0.29) [a]

Mean (SD); $\mu g/10^9$ bacterial cells.
[a]Significant difference with PBS (non cation); ($P < 0.05$).

Bacterial adhesion to epithelial cells

The number of bacteria adhering per epithelial cell is shown in Table 2. Adhesion was high for both species, although it was greater for *P. intermedia* than *P. nigrescens*.

Pretreatment with human serum proteins inhibited adhesion of both species, and the presence of zinc or copper during treatment with proteins markedly inhibited the adhesion in a concentration-dependent manner.

Table 2. Bacterial adhesion to epithelial cells

Treatment of bacterial cells	*P. intermedia* ATCC25611	*P. nigrescens* ATCC33563
Background (already attached)	18 (6)	18 (6)
Non treatment (Harvested only)	340 (101)	167 (22)
Human serum coated	269 (77)	136 (10) [a]
Human serum coated with 0.5 mM $ZnCl_2$	72 (21) [a,b]	42 (3) [a,b]
Human serum coated with 1 mM $ZnCl_2$	28 (7) [a,b,c]	21 (3) [a,b]
Human serum coated with 0.5 mM $CuCl_2$	68 (26) [a,b]	41 (2) [a,b]
Human serum coated with 1 mM $CuCl_2$	28 (4) [a,b,c]	21 (3) [a,b]

Mean (SD): number of bacterial cells adhering to epithelial cell.
[a]Significant difference with non treatment ($P < 0.05$).
[b]Significant difference with human serum coated ($P < 0.05$).
[c]Significant difference with serum coated with each 0.5 mM cation ($P < 0.05$).

Hemagglutination assay

The effect of bacterial suspension dilution on hemagglutination inhibition is shown in Table 3. Hemagglutination was inhibited when both species of bacteria were treated with human serum proteins and was strongly inhibited in the presence of zinc or copper during protein treatment. Zinc and copper cations had a concentration-dependent effect on hemagglutination, which suggests that they affect hemagglutination in a similar manner to bacterial adhesion.

Table 3. Hemagglutination assay

Treatment of bacterial cells	*P. intermedia* ATCC25611	*P. nigrescens* ATCC33563
Non treatment (harvested only)	40	15
Human serum coated	20	5
Human serum coated with 0.5 mM $ZnCl_2$	5	2.5
Human serum coated with 1 mM $ZnCl_2$	2	1
Human serum coated with 0.5 mM $CuCl_2$	5	2.5
Human serum coated with 1 mM $CuCl_2$	2	1

The dilutions of bacterial suspensions that inhibited the hemagglutination activity of the bacterial strains.

SDS-PAGE and fluorography of attached human serum proteins

Fluorographic images of the SDS-PAGE profiles of proteins adsorbed to *P. intermedia and P. nigrescens* are shown in Fig. 1. For *P. intermedia*, eight major bands, corresponding to attached human serum proteins, with approximate molecular masses of 67, 56, 49, 45, 34, 25, 22, and less than 20 kDa, were observed. The presence of calcium increased the density of all of the protein bands, especially that migrating at less than 20 kDa, whereas zinc or copper increased the density of only the 67, 56, 45, 25, and 22 kDa protein bands. For *P. nigrescens*, eight major bands were also seen, corresponding to attached human serum proteins with molecular masses of approximately 82, 75, 67, 56, 49, 45, 25, and 22 kDa. The presence of calcium enhanced the intensity of all these bands, while zinc enhanced only the 67, 56, 45, and 22 kDa bands.

Each species showed a different pattern of attached human serum protein bands. Although zinc or copper enhanced the binding of some protein component bands, we did not identify specific bands that bound zinc or copper selectively.

Cation-binding components on bacterial cells

Cation-binding components in human serum proteins and in isolates from bacterial surfaces were identified in cation column elutes by silver staining of SDS-PAGE gels, as shown in Figs 2–4. Several protein components were identified with affinity for each of the cations. Although the amounts of proteins attached to each cation differed, it was difficult to identify specific cation-binding human serum proteins.

For the surface proteins from *P. intermedia*, we detected no iron-binding proteins, 11 zinc-binding, 12 copper-binding, 4 cerium-binding, and 12 nickel-binding proteins. Proteins of 100 and 21.2 kDa bound zinc specifically, and proteins of 31 and 26.5 kDa were specific for copper (Fig. 3, arrows).

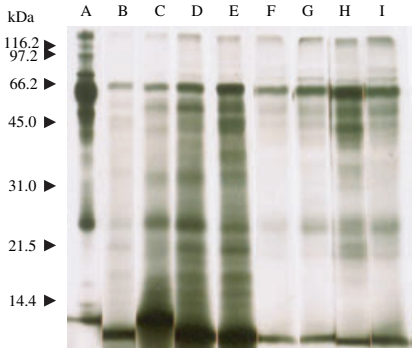

Fig. 1. Analysis of attached human serum proteins by SDS-PAGE and fluorography. A, human serum proteins. B, human serum proteins attached to *P. intermedia* ATCC25611. C, *P. intermedia* ATCC25611 by the presence of $CaCl_2$. D, *P. intermedia* ATCC25611 by the presence of $ZnCl_2$. E, *P. intermedia* ATCC25611 by the presence of $CuCl_2$. F, human serum proteins attached to *P. nigrescens* ATCC33563. G, *P. nigrescens* ATCC33563 by the presence of $CaCl_2$. H, *P. nigrescens* ATCC33563 by the presence of $ZnCl_2$. I, *P. nigrescens* ATCC33563 by the presence of $CuCl_2$.

Among the surface proteins from *P. nigrescens*, we detected no iron-binding proteins, 8 zinc-binding, 14 copper-binding, 1 cerium-binding, and 10 nickel-binding proteins. The 34 kDa protein bound zinc specifically, and the 80, 29, 25, and 23.5 kDa proteins showed copper-specific binding (Fig. 4, arrows).

Discussion

The adhesion of pathogenic bacteria to oral tissues in the gingival sulcus is believed to initiate the pathogenesis of periodontal disease (4, 6). Evidence suggests that proteins or other components of saliva or GCF can influence the ability of such bacteria to adhere to oral surfaces and thus affect the initiation and/or progression of disease (12).

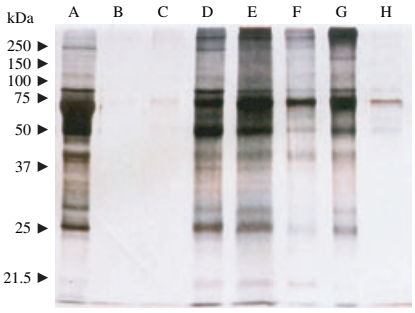

Fig. 2. Cation-binding components in human serum proteins. A, human serum proteins. B, non cation-binding chelating column (control). C, iron-binding chelating column. D, zinc-binding chelating column. E, copper-binding chelating column. F, cerium-binding chelating column. G, nickel-binding chelating column. H, aluminum-binding chelating column.

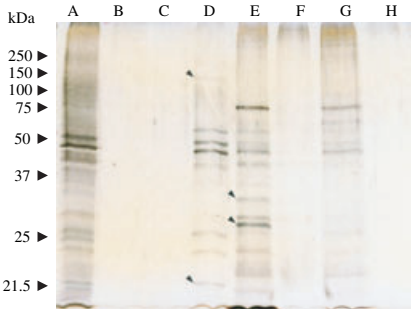

Fig. 3. Cation-binding components from the surface of *P. intermedia* ATCC25611. A, human serum proteins. B, non cation-binding chelating column (control). C, iron-binding chelating column. D, zinc-binding chelating column. E, copper-binding chelating column. F, cerium-binding chelating column. G, nickel-binding chelating column. H, aluminum-binding chelating column. Specific zinc- or copper-binding bands are indicated by arrows.

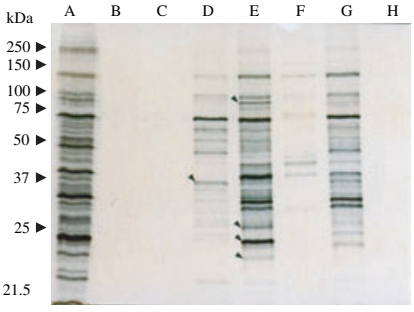

Fig. 4. Cation-binding components from the surface of *P. nigrescens* ATCC33563. A, human serum proteins. B, non cation-binding chelating column (control). C, iron-binding chelating column. D, zinc-binding chelating column. E, copper-binding chelating column. F, cerium-binding chelating column. G, nickel-binding chelating column. H, aluminum-binding chelating column. Specific zinc- or copper-binding bands are indicated by arrows.

We investigated the attachment of human proteins to two species of *Prevotella* using an artificial GCF composed of human serum proteins in PBS, and examined the effects of attached proteins on bacterial adhesion and hemagglutination. As phosphates may influence the effect of metal cations, we used a solution with 5 mM phosphate, which is much lower than that found in saliva. Cation-bridge binding appears to play an important role in the attachment of human serum proteins to bacterial cell surfaces. Different amounts of attached proteins may be dependent on the depositing additional protein activity of each of the cations, especially zinc, copper, and cerium (5). Therefore, the effect of these cations on promoting the attachment of proteins to the surfaces of *Prevotella* species should be greater than their effect on protein precipitation. Hydrophobic binding and lectin-ligand binding did not appear to be important to the attachment mechanism, although both can affect bacterial adhesion and aggregation on oral tissues (3). This indicates that protein attachment to *Prevotella* spp. may have a different mechanism to bacterial epithelial adhesion and aggregation. The results from the bacterial heat treatment experiments suggest that the protein components of the bacterial cell surface play an important role in attachment.

The presence of zinc or copper during bacterial treatment with proteins strongly inhibited adhesion to epithelial cells and hemagglutination of both species. This may be caused either by marked masking of adhesion sites on bacterial surfaces or by protein-induced aggregation. In the latter case, the clumping of *Prevotella* spp. reduces adhesion and the unattached bacterial cells are easily removed by washing (10).

We postulated that proteins with high affinity for zinc or copper in human serum and/or on the surface of *Prevotella* spp. play an important role in the inhibition of adhesion and hemagglutination. By labeling human serum proteins and following them by fluorographically, we were able to track their attachment to bacteria in the presence of protein components of the bacterial cell surface and cytoplasm. As specific zinc- or copper-binding human serum protein bands were not identified either by fluorography nor on cation-chelating columns, increasing the density of these protein bands may be attributable to the protein precipitation induced by each of the cations. In contrast, different cation-binding proteins, including those specific for zinc or copper, appeared to be present on the surface of each bacterial species. The effect of cations on the attachment of human serum proteins may be dependent upon bacterial cell surface components. Additionally, it seems probable that cation-binding proteins might play a role in specific binding, especially in the cases of zinc and copper.

In conclusion, cations, especially zinc and copper, markedly enhanced the attachment and accumulation of human serum proteins on the strains tested and inhibited their adhesion and hemagglutination.

Accordingly, zinc and copper might be useful in the prevention or inhibition of periodontal disease. A study of cation-binding proteins on bacterial cell surfaces and the cation-bridge binding system, including specific binding factors, would be useful for clarifying the processes of bacterial adhesion and aggregation.

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