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Inhibitory effect of serum globulins on the adhesion of *Prevotella nigrescens* to hydroxyapatite

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Abstract: *Prevotella nigrescens* **ATCC 25261 cells adhere well to protein-blocking hydroxyapatite (HA) which mimics a root surface in the periodontal pocket treated with proteases such as trypsin, proteinase K, chymotrypsin and papain. This study was done to clarify the inhibitory effect of α- and β- serum globulins on the adhesion of *P. nigrescens* ATCC 25261 cells to trypsin-treated HA. The inhibitory effect was found to be caused by the α1-antitrypsin (α1-AT) of α-globulin and the low-density lipoprotein (LDL) of β-globulin under experimental conditions. The most effective inhibition of α1-AT on *P. nigrescens* ATCC 25261 cell attachment to HA was achieved when α1-AT-treated trypsin was used. The most effective inhibition of LDL occurred when trypsin-treated HA was treated with LDL. Apo-transferrin (TF) and holo-TF, which are β-globulins, did not affect the attachment of *P. nigrescens* ATCC 25261 cells to trypsin-treated HA. (J. Oral Sci. 45, 11-16, 2003)**

Key words: *Prevotella nigrescens*; hydroxyapatite; trypsin; adhesion; inhibition.

Introduction

Oral gram-negative anaerobic rods such as *Porphyromonas gingivalis* and *Prevotella intermedia* may

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be potential etiologic agents of severe periodontitis (1,2). The periodontal pocket has been assumed to be the primary habitat of these bacteria (3).

The *P. intermedia* group consists of three species, *P. intermedia, P. nigrescens* and *P. pallens*, that are indistinguishable by routine phenotypic characteristics (4-6). Different species within the *P. intermedia* group may exhibit differences in colonization pattern and pathogenic potential. *P. intermedia* has rarely been isolated in children, unlike *P. nigrescens* and *P. pallens* (7-10). *P. intermedia* is mainly associated with periodontally diseased sites, whereas *P. nigrescens* has also been isolated from the oral cavity of periodontally healthy adults and children (7,8,11). The newly described *P. pallens* also constitutes a common member of the oral microflora in early childhood (12).

Bacterial adherence to oral tissue surfaces is observed in the first stage of dental caries and periodontal disease (13,14). The mechanisms which mediate bacterial attachment are thought to be both specific (such as lectin-ligand binding) and non-specific (such as hydrophobic effects). Specific binding results from stereochemical interactions involving proteinaceous ligands, called "adhesins", on the bacterial surface and complementary molecules, called "receptors", on the host tissue (15-18).

P. nigrescens ATCC 25261 cells adhere to bovine serum albumin (BSA)-blocking hydroxyapatite (HA) which mimics a root surface in the periodontal pocket treated with citrate, but do not adhere well to HA that has not been treated with citrate (19). Citrate is also an essential factor for *P. nigrescens* ATCC 25261 cell attachment to apatitic surfaces, and the glycoproteins of the *P. nigrescens* ATCC 25261 cell surface component function as adhesins (20). Likewise, proteases, such as trypsin, chymotrypsin,

proteinase K and papain also promote bacterial attachment, and are inhibited by the α1-AT of serum globulin (21).

In this study, we examined the inhibitory effects of some serum proteins other than α1-AT on the adhesion of *P. nigrescens* ATCC 25261 cells to the surface of BSA-blocking HA that mimics the root surface, with reference to the findings that the adhesion of these cells occurred subsequent to adsorption of trypsin to the BSA-blocking HA, and was inhibited in the presence of α1-AT.

Materials and Methods

Bacterial strain and culture conditions

P. nigrescens ATCC 25261 was obtained from the culture collection of our laboratory. *P. nigrescens* ATCC 25261 was preincubated in GAM broth (Nissui, Japan) in an anaerobic jar for 24 h at 37°C in an atmosphere of 95% N_2 and 5% CO_2. The fresh bacterial cells were then inoculated into GAM broth supplemented with 740 kBq of [^{3}H] thymidine (ICN Biochemicals, CA, USA) per ml. Bacteria used in the adhesion assay were grown to the early stationary phase at 37°C under anaerobic conditions (BBL GasPak Anaerobic System, MD, USA). Bacterial cells were harvested by centrifugation (3 min, $10000 \times g$, room temperature) and washed twice with buffered KCl (0.05 M KCl containing 1 mM K_2PO_4, 1 mM $CaCl_2$ and $MgCl_2$ at pH 6.2). The washed cells were then suspended in BSA-KCl (buffered KCl supplemented with 5 mg per ml of BSA, Sigma Chemical Co., MO, USA). The suspensions were adjusted to a level of 6×10^8 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 HA beads (Nihon Chemical, Japan) treated with trypsin (Sigma Chemical, MO, USA). Before the assay, 5 mg of beads were equilibrated overnight in buffered KCl at room temperature (RT). After treating HA with BSA-KCl for 30 min at RT to block any uncoated bead surfaces (22), the beads were then washed twice with buffered KCl and incubated with an adequate concentration of trypsin solution for 30 min at RT. The beads were washed again, the liquid was removed, and then the beads were incubated with ^{3}H-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 adherent cells was determined by direct scintillation counting (LSC-5200: Aloka, Japan). The number of bacterial cells was calculated from specific activity compared to a standard that contained 3×10^7 ^{3}H-labeled cells in 25 μl at $OD_{550nm} = 0.2$.

The influence of the presence of serum proteins (BSA-A7638: globulin-essentially free, BSA-A8022: remainder mostly globulins, BSG-G9762 (bovine serum globulin): predominantly α- and β-globulins) was also studied in the experimental system (30 min, RT). The inhibitory effect of α- and β- globulin proteins on the attachment of *P. nigrescens* ATCC 25261 to trypsin-treated HA was also studied by mixing (30 min, RT) each selected compound with *P. nigrescens* ATCC 25261 cells and then adding this mixture to trypsin-treated HA.

Results

Effect of trypsin concentration on *P. nigrescens* ATCC 25261 cell attachment to HA

The attachment of *P. nigrescens* ATCC 25261 cells to BSA-blocking HA treated with increasing amounts of trypsin, ranging from 0 to 2.0 mg/ml, was tested. The attachment was dose-dependent up to a trypsin concentration of 0.1 mg/ml (Fig. 1).

Effect of treatment of trypsin with BSA-A8022, BSA-A7638 and BSG-G9762 on *P. nigrescens* ATCC 25261 cell attachment

Of the proteins tested, only two produced significant inhibition of trypsin. BSG-G9762 was a strong inhibitor. The number of bacteria binding to HA was not affected by treatment of trypsin with BSA-A7638 (Figs. 2 and 3).
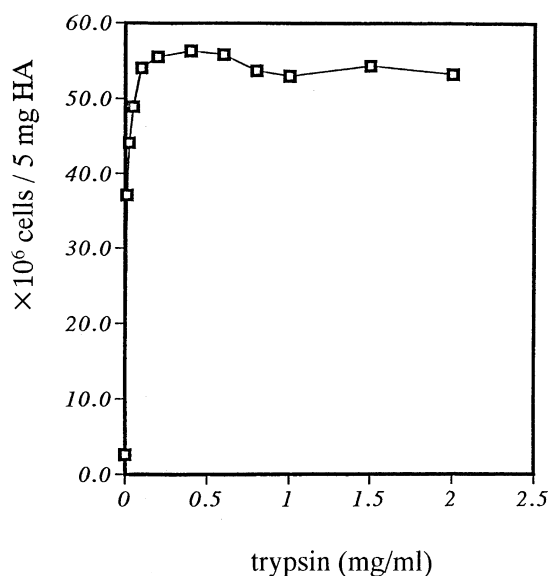

Fig. 1 Effect of trypsin concentration on *P. nigrescens* cell attachment to HA.

Inhibitory effect of trypsin and α1-AT treatment order on *P. nigrescens* ATCC 25261 cell attachment to BSA-blocking HA

The inhibitory effects of treatment procedures using α1-antitrypsin (α1-AT) were tested.

(1) α1-AT-treated HA was treated with trypsin

(2) trypsin-treated HA was treated with α1-AT

(3) HA treated with trypsin was incubated with α1-AT

The most effective inhibition of *P. nigrescens* ATCC 25261 cell attachment was obtained under condition (3); 1.25 mg/ml α1-AT-treated trypsin was sufficient to produce the maximum inhibitory effect (Figs. 4 and 5).
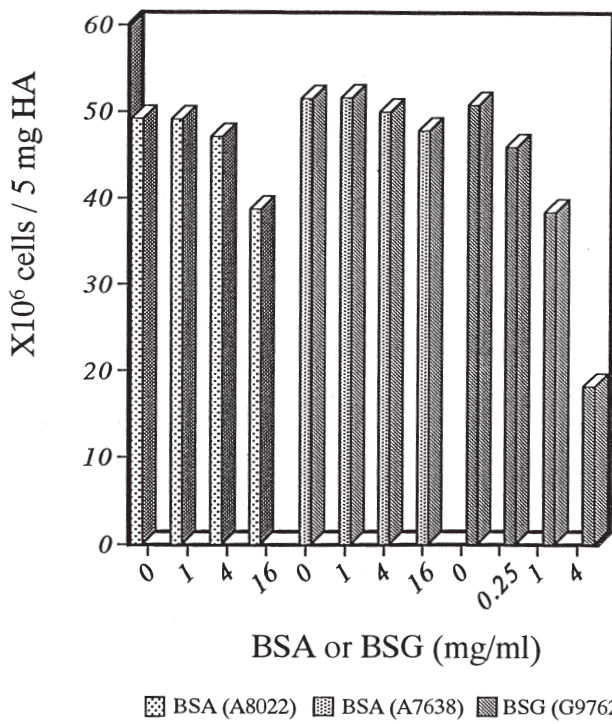
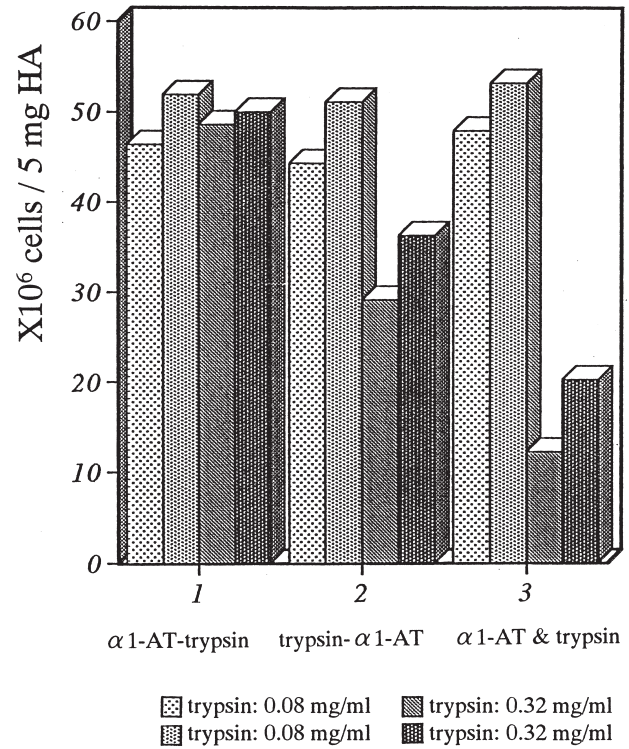Fig. 2 Effect of treatment of trypsin with BSA or BSG on *P. nigrescens* cell attachment to HA.

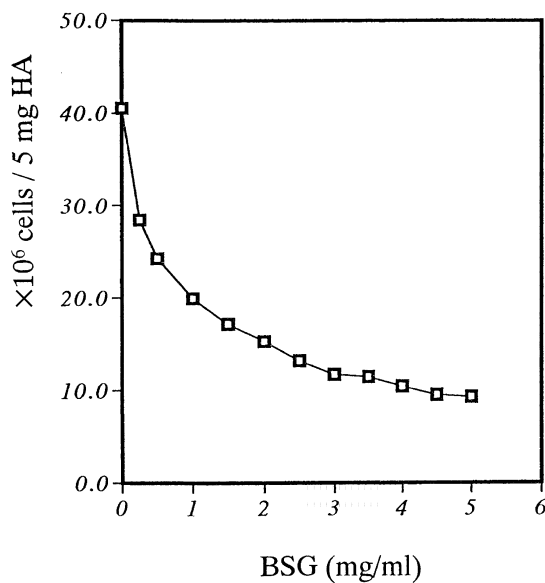Fig. 4 Effect of α1-AT treatment procedure on *P. nigrescens* cell attachment to HA.

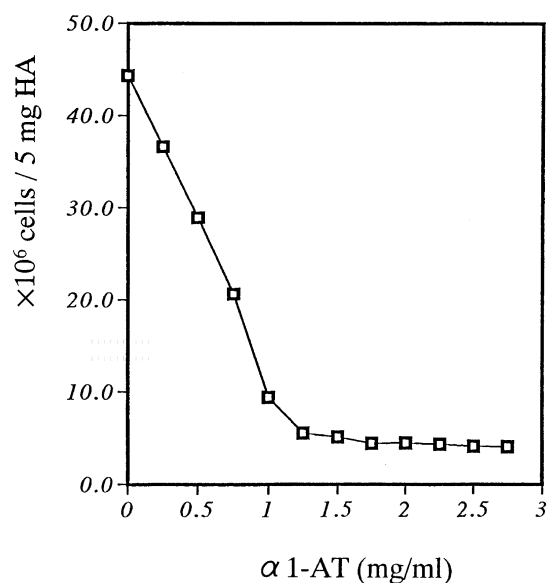Fig. 3 Inhibitory effect of BSG on *P. nigrescens* cell attachment to trypsin-treated HA.

Fig. 5 Inhibitory effect of α1-AT on *P. nigrescens* cell attachment to trypsin-treated HA.

Inhibitory effect of trypsin and LDL treatment order on *P. nigrescens* ATCC 25261 cell attachment to BSA-blocking HA

The inhibitory effects of treatment procedures using low-density lipoprotein (LDL) were tested.

(1) LDL-treated HA was treated with trypsin

(2) trypsin-treated HA was treated with LDL

(3) HA treated with trypsin was incubated with LDL

The most effective inhibition of *P. nigrescens* ATCC 25261 cell attachment was obtained under condition (2); 0.1 mg/ml LDL-treated trypsin-treated HA was sufficient to produce the maximum inhibitory effect (Figs. 6 and 7).

Effect of TF treatment of trypsin-treated BSA-blocking HA on *P. nigrescens* ATCC 25261 cell attachment

Apo-transferrin (TF) and holo-TF were tested. Neither affected the attachment of *P. nigrescens* ATCC 25261 cells to trypsin-treated BSA-blocking HA (Fig. 8).

Discussion

Connective tissue destruction is a major feature of chronic periodontitis, and proteolytic enzymes are believed to play a significant role in its pathogenesis. The appearance of proteases in gingival crevicular fluid (GCF) may therefore be an indicator of the disease severity (23,24).

Proteolytic enzymes are known to play an important role in inflammatory conditions such as periodontal disease. The main sources of host-derived proteases in periodontal tissues are believed to be the connective tissue and/or inflammatory cells (25). A number of bacteria from the subgingival flora have been associated with the progression of periodontal disease (26), and some of them produce proteolytic enzymes. The most abundant bacterial proteases produced are trypsin-like proteases and dipeptidyl
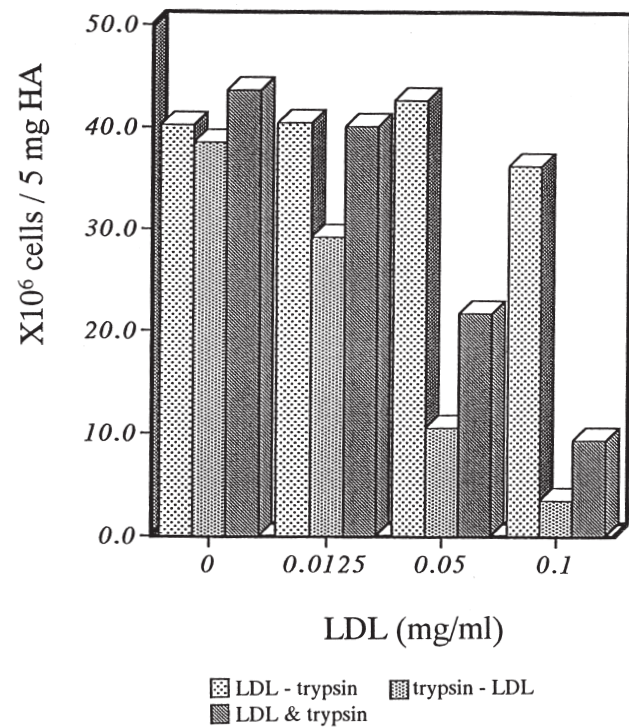
Fig. 7 Inhibitory effect of LDL on *P. nigrescens* cell attachment to trypsin-treated HA.

Fig. 6 Inhibitory effect of trypsin treatment order with LDL on *P. nigrescens* cell attachment to HA.

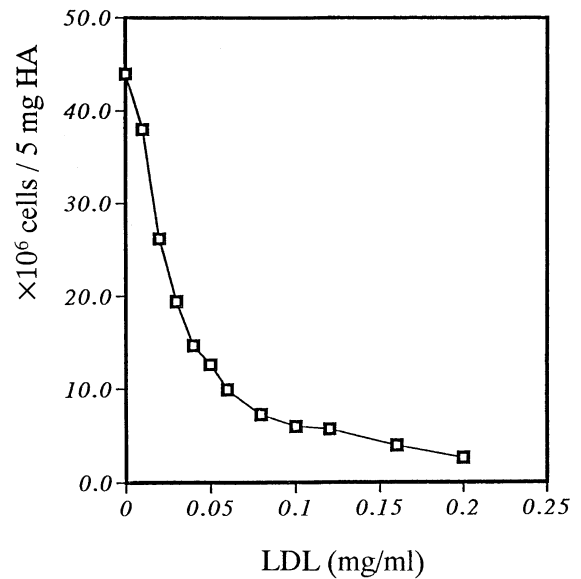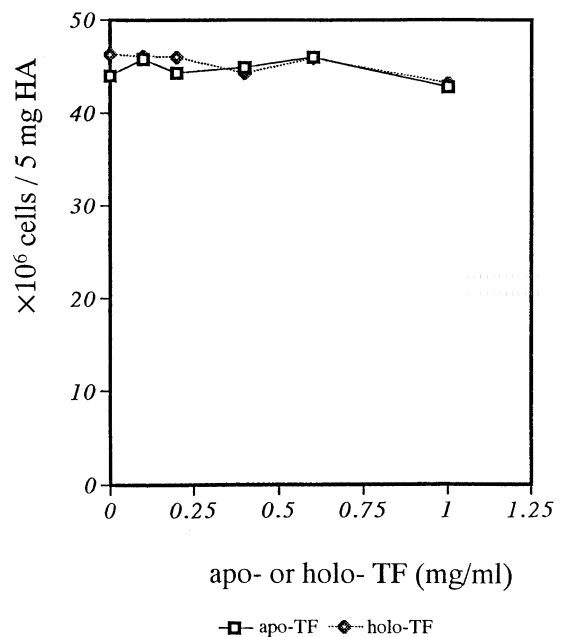Fig. 8 Effect of TF treatment of trypsin-treated HA on *P. nigrescens* cell attachment.

peptidases (DPP), which are thought to mediate tissue destruction and disturb host defense mechanisms (27,28). These proteases have been shown to promote inflammatory reactions, whereas their inhibitors may act as immuno-suppressive agents (24).

The attachment of oral bacteria to oral hard surfaces is undoubtedly important in the etiology and progression of oral disease. *P. nigrescens* ATCC 25261 cells adhere to HA treated with proteases such as trypsin, proteinase K, chymotrypsin and papain (21). The purpose of this study was to clarify the inhibitory effect of α- and β- serum globulins on the adhesion of *P. nigrescens* ATCC 25261 cells to trypsin-treated HA. The influence of the presence of BSA-A8022, BSA-A7638 and BSG-G9762 was also studied. The inhibitory effect on *P. nigrescens* ATCC 25261 cell attachment to trypsin-treated HA was much higher with BSG-G9762 than with BSA-A8022. Although these two serum proteins contain a globulin, the inhibitory effect caused by the α1-AT of α-globulin and LDL of β-globulin was considered. The most effective inhibition by α1-AT of *P. nigrescens* ATCC 25261 cell attachment to HA was shown to occur when α1-AT-treated trypsin was used. The most effective inhibition by LDL was found when trypsin-treated HA was treated with LDL. It was observed that α1-AT inhibited trypsin- and LDL-covered receptors which are present on the trypsin-treated HA surface. However, apo-TF and holo-TF, which are β-globulins, did not affect the attachment of *P. nigrescens* ATCC 25261 cells to trypsin-treated HA.

The proteins of GCF are assumed to be derived from serum and host epithelial and connective tissues, as well as from subgingival microbes. Most proteins that have been identified in GCF are of serum origin, such as albumin, immunoglobulins, complement components, transferrin, fibrinogen, and protease inhibitors (29-33). The main serum protease inhibitors, α1-AT and α2-macroglobulin (accounting for approximately 90% of the serum's inhibitory capacity), are thought to play a role in the protection of periodontal tissues (34).

Our findings suggest that *P. nigrescens* ATCC 25261 may adhere to the root surface when exposed to trypsin in gingival fluid derived from host and other bacteria. This adhesion also may be inhibited by increased globulins in relation to the increase of gingival fluid as a result of the host's immune reaction against the progression of periodontal infections.

Acknowledgments

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