Citrate promotes attachment of *Prevotella nigrescens* (intermedia) ATCC 25261 to hydroxyapatite

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(Received 26 September 1997 and accepted 20 February 1998)

**Abstract:** Large quantities of *Prevotella nigrescens* (intermedia) ATCC 25261 (*P. nigrescens*) cells adhere to hydroxyapatite (HA) treated with citrate, but do not adhere experimental pellicle prepared from human saliva. To determine the nature of the citrate responsible for promoting *P. nigrescens* cell adhesion, the duration and frequency of citrate treatment of HA and the inhibitory effect of other carboxylates were tested. The citrate rapidly adhered to HA beads in less than 15 min. With a lower concentration (0.4 mM) of citrate, four treatments of HA were required to promote the maximum adherence to *P. nigrescens* cells. Citrate-enhanced *P. nigrescens* cell adherence to HA beads was also inhibited in the presence of cis-aconitate, oxaloacetate and oxalacetate. It was also found that *P. nigrescens* cells heated to 65°C or higher for 5 min could no longer become attached to citrate-treated HA. These data suggest that citrate is one of the essential factors responsible for *P. nigrescens* cell attachment to apatitic surfaces, and that *P. nigrescens’* adhesion to citrate is extremely heat-sensitive. (J. Oral Sci. 40, 65-69, 1998)

**Key words:** *Prevotella nigrescens* (intermedia); citrate; bacterial adsorption; hydroxyapatite.

**Introduction**

The oral bacteroidea have been recognized as prominent constituents of mature dental plaque (1-4). Although samples taken from the surface of freshly cleaned teeth contain an extremely low concentration of bacteroidea, samples removed from the diseased sites of patients with moderate or severe periodontitis contain many Bacteroidea spp (5, 6). Some species, such as *Prevotella loeschei* and *Porphyromonas gingivalis*, may be potential etiologic agents of severe periodontitis (6, 7). Others, like *Prevotella loeschei*, occur in similar numbers in both healthy persons and patients with moderate periodontitis (5). Regarding plaque bacteria, attachment to the tooth surface is the initial event in dental plaque formation (8). The mechanisms which mediate bacterial attachment are thought to be specific and non-specific forces. Specific binding results from stereochemical interactions involving proteinaceous ligands, called “adhesins,” on the bacterial surfaces and complementary molecules, called “receptors,” on the host tissue (9, 10). Many adhesins are associated with fimbriae or pili of the cell surface, which bind to carbohydrate receptors (9, 11-13).

It has been reported that during puberty children have a higher incidence of gingivitis than either younger children or adults (14-16). A similar increase in gingivitis has been reported during pregnancy (17-21). Several studies have suggested that gingivitis during pregnancy is associated with higher levels of estradiol and progesterone (19, 22, 23). It has also been demonstrated that hormonal changes may affect the composition of the subgingival flora (22, 24). *P. nigrescens* is able to substitute menadione with estradiol and progesterone (23, 25).

It has also been reported that *P. nigrescens*, the *Treponema* sp. and the *Fusobacterium* sp. are found in all plaque samples of patients with ANUG. Also *P. nigrescens* accounts for 24% of the cultural count (26). These findings suggest that *P. nigrescens* may play an important role in the occurrence of periodontitis. Nevertheless, it is not yet known how *P. nigrescens* adheres to hard surfaces.

The purpose of the present study was to clarify the role of citrate in the adhesion of *P. nigrescens* cells to HA beads used to mimic a hard tooth surface.

**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 anaerobic conditions (BBL GasPak Anaerobic System, Becton Dickinson...
Microbiology Systems, MD, USA). Bacterial cells were harvested by centrifugation, washed twice and suspended in buffered KCl (0.05 M KCl containing 1 mM K2HPO4, 1 mM KH2PO4, 1 mM CaCl2, and 0.1 mM MgCl2 at pH 6.2) supplemented with 5 mg per ml of bovine serum albumin (Sigma Chemical Co., MO, USA). The suspensions were adjusted to a level of $6 \times 10^8$ bacteria per ml based on a standard curve relating optical density (550 nm) to the number of bacterial cells, as determined by microscopic counting.

Adhesion assays

Bacterial binding to HA was studied with the use of citrate (trisodium citrate dihydrate: Wako Pure Chemical Industries, Ltd., Japan)-treated HA beads. Bacterial attachment was determined from experimental pellicles formed from human whole saliva on HA beads (Nihon Chemical Co., Japan). Before the assay, the beads were equilibrated overnight in buffered KCl at room temperature. Experimental pellicles were prepared by treating 5 mg of equilibrated HA beads with 125 μl of human whole saliva on microtitration plates. The mixtures were incubated for one h at room temperature with continuous rotation (6 rpm). The beads were then washed twice with buffered KCl, and treated for 30 min with 5 mg/ml of bovine serum albumin in buffered KCl (BSA-KCl) to block any uncoated bead surfaces (27). The liquid was then removed, and the beads were incubated with an adequate concentration of citrate solution. Similarly, the liquid was removed, and the 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 room temperature, the beads were washed twice with buffered KCl, and transferred to scintillation vials. The number of cells attached was determined by direct scintillation counting (Model 2000CA; Packard Instrument Co., Inc., CT, USA). The influence of the presence of other carboxylates (cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, oxalacetate and oxalacetinate) on P. nigrescens attachment to citrate-treated HA was also studied by mixing each selected compound with P. nigrescens cells then adding this mixture to citrate-treated HA. An additional experiment was also performed where samples of [3H]-thymidine-labeled P. nigrescens cells were incubated at 56°C, 65°C and 80°C for varying lengths of time prior to incubation with citrate-treated HA. All assays were conducted in duplicate, and most experiments were performed at least twice.

Preparation of saliva

Samples of whole unstimulated saliva were collected from a single adult donor and placed in ice-chilled containers. The samples were heated successively to 56°C for 30 min to inactivate degradative enzymes, clarified by centrifugation at 2,000 × g for 20 min, and filtered with a membrane (0.45 μm and 0.20 μm; Nihon Millipore Kogyo K. K., Japan). The samples were then dialyzed for 3 days at 4°C in distilled water containing 0.004 % NaN3.

The liquid was changed three times. The dialyzed samples were lyophilized and stored at -80°C until used.

Results

Attachment of P. nigrescens cells to saliva-treated HA (SHA) and citrate-treated SHA

Treatment of HA beads with only saliva did not promote the attachmet of P. nigrescens cells, whereas citrate-treated SHA produced a remarkable increase in the attachment of P. nigrescens cells. The attachment was dose-dependent up to a citrate concentration of 4 mM (Fig. 1).

Fig. 1 Adsorption of P. nigrescens to citrate-treated SHA and HA.

Fig. 2 Adsorption of P. nigrescens to HA-beads treated with various concentration of citrate.
Effect of duration of citrate-treatment of HA on the attachment of *P. nigrescens* cells to HA

The cell count was the same for bacteria which bound to HA beads treated with citrate for various durations (15 min, 30 min, 45 min and 60 min) (Fig. 2). It was therefore concluded that 15 min of citrate treatment of HA was enough time to produce a maximum effect.

Effect of frequency of citrate treatment of HA on the attachment of *P. nigrescens* to HA

The number of bacteria binding to HA beads increased in proportion to the frequency of treatment with citrate. Even when the concentration of citrate was relatively low (0.4 mM, 6 times), the maximum number of *P. nigrescens* cells could become attached to the HA beads (Fig. 3, 4).

Effect of other carboxylates on *P. nigrescens* attachment to citrate-treated HA

Of those carboxylates tested, only three produced significant inhibition of *P. nigrescens* attachment. Oxalosuccinate, oxaloacetate and cis-aconitate were strong inhibitors (Fig. 5). The other carboxylates did not affect the attachment of *P. nigrescens* to citrate-treated HA.
Effect of heat treatment of *P. nigrescens* cells on their ability to attach to citrate-treated HA

*P. nigrescens* cells heated to 65°C or higher (80°C) for 5 min could not become attached to citrate-treated HA beads. When heated at 56°C, *P. nigrescens* cells had only a slight ability to bind to HA (Fig. 6-8).

**Discussion**

It is well recognized that dental caries and periodontal diseases are infections caused by bacterial plaque accumulating on tooth surfaces. Many efforts to identify the mechanisms involved in the formation of such dental plaques have revealed that bacterial attachment to tooth surfaces is a specific process, and the attachment is the first step of the bacterial colonization of teeth. The majority of oral bacteria display marked tissue tropisms. It has become evident that bacteria possess a highly developed recognition system which can identify and interact with specific macromolecules present on tooth surfaces (28).

The oral bacteroides are a prominent constituent of mature dental plaque. Of these oral bacteroides, *P. nigrescens* has been isolated in the female gingivitis of puberty and pregnancy, and also in high numbers in plaque samples of patients with ANUG.

It has been shown that the citric acid concentrations in both serum and saliva in clinically healthy subjects are approximately 0.13 mM and 0.04 mM, respectively. Moreover, citric acid is significantly more concentrated in serum from periodontal patients than in that from clinically healthy subjects (29, 30). Citrate is an important part of the TCA cycle, but some periodontal diseases are thought to be related to hypercitricemia (31).

One of the most remarkable findings in the present study was that the citrate which adheres to HA bead surfaces interacts with *P. nigrescens* cells. These interactions seem to suggest that a lectin-like bacterial adhesion mechanism operates between the bacterial cell surfaces and citrate molecules adhered to HA. The other citrates such as calcium salt, potassium salt, magnesium salt, lithium salt and ammonium salt exhibited similar effects (unpublished observations). The “adhesin” of *P. nigrescens* cell surfaces has also been shown to be very sensitive to heat. Citrate is generally found in serum, saliva and periodontal pocket exudate from both patients with periodontal disease and clinically healthy subjects.

**Conclusion**

Several mechanisms are involved in the adherence of oral bacteria to the surfaces of tooth and oral mucosa. In this study, it has been demonstrated that citrate is one of the essential factors in *P. nigrescens* cell attachment to apatitic surfaces, and that the adhesin which interacts with citrate is extremely heat sensitive. This interaction is also strongly inhibited in the presence of cis-aconitate, oxaloacetate and oxalsuccinate.

**Acknowledgments**

This research was supported in part by a grant from the Satoh Research Fund.

**References**

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