Collagen-binding activity of *Prevotella intermedia* measured by a microtitre plate adherence assay

Daniel Grenier

Tel: +1 418 656 7341. Fax: +1 418 656 2861.
e-mail: Daniel.Grenier@greb.ulaval.ca

Groupe de Recherche en
Ecologie Buccale, Faculté
de Médecine Dentaire,
Université Laval, Sainte-
Foy, Québec, Canada
G1K 7P4

The ability of *Prevotella intermedia* to bind type I collagen was investigated. A simple method in which bacterial cells were allowed to attach to collagen-coated microtitre plate wells was used to characterize the activity. All strains of *P. intermedia* tested, as well as those of the closely related species *Prevotella nigrescens*, showed a capacity to attach to the collagen film. Exponential-phase cultures of *P. intermedia* demonstrated a greater binding capacity than older cells. Attachment to the collagen film was inhibited by the presence of EDTA, type I and IV collagen, denatured collagen (gelatin), fibrinogen or fibronectin. Pretreatment of bacterial cells with heat (60 °C, 30 min) or proteinase K also inhibited the binding. The collagen-binding activity could be solubilized from the bacterial cell surface by incubation with Zwittergent 3-14, a zwitterionic detergent. The collagen-binding capacity of *P. intermedia* demonstrated in the present study represents a mechanism of colonization allowing these bacteria to attach to a tissue matrix.

Keywords: *Prevotella*, periodontopathogen, periodontal disease, collagen-binding activity, bacterial adherence

INTRODUCTION

Prevotella intermedia and *Prevotella nigrescens*, both formerly designated *P. intermedia* (Shah & Gharbia, 1992), may be isolated from subgingival sites of healthy patients and of those suffering from various forms of periodontal diseases (Slots & Listgarten, 1988; Teanpaisan *et al.*, 1995), which suggests that they may be opportunistic pathogens. Virulence factors that may be important for the virulence of these bacteria include hydrolytic enzymes and host-binding activities (Holt & Bramanti, 1991).

The ability of periodontopathogens to attach to oral surfaces is believed to be a critical step in the initiation of the pathogenic process of periodontal diseases. Previous reports have shown that *P. intermedia* can attach to epithelial cells, erythrocytes and other oral bacteria (Devine & Handley, 1989; Leung *et al.*, 1989). Host proteins, including fibrinogen, laminin and IgG, are also bound by *P. intermedia* (Kalfas *et al.*, 1992; Labbé & Grenier, 1995; Lantz *et al.*, 1985). In the present investigation, the ability of *P. intermedia* to bind collagen was demonstrated and characterized using a simple microtitre plate adherence assay.

METHODS

Bacteria and growth conditions. *P. intermedia* strain BMH, obtained from B. C. McBride (University of British Columbia, Vancouver, Canada), was used in most experiments. This isolate was identified in a previous study (Jansen *et al.*, 1995) using the Rapid ID 32A system (Bio-Mérieux) and multilocus enzyme electrophoresis. Other oral bacterial strains, listed in Table 1, were also tested for their collagen-binding activity. Except for *Treponema denticola*, bacteria were grown anaerobically [$N_2/H_2/CO_2$ (80:10:10, by vol.)] at 37 °C in brain heart infusion broth (BBL Microbiology Systems) supplemented with haemin (10 µg ml⁻¹) and vitamin K (1 µg ml⁻¹). *T. denticola* was cultivated under the same conditions but in the oral spirochaete medium as previously described by Leschine & Canale-Parola (1980). Unless indicated, bacteria were grown to early stationary phase (approximately 24 h), harvested by centrifugation, washed twice in PBS (0.145 M NaCl; 0.05 M sodium phosphate), pH 7.0, and suspended in the same buffer to an OD₆₆₀ of 2.0.

Microtitre plate collagen-binding assay. Type I acid-soluble collagen (calf skin; Sigma) was prepared in 0.02% acetic acid, to a concentration of 2 mg ml⁻¹, by continuous stirring at 4 °C for 16 h. Wells of a flat-bottomed microtitre plate (Immulon 1;

Dynatech Laboratories) were filled with the collagen solution (100 µl), and the plate was incubated overnight at room temperature. Control wells were coated with 1% (w/v) BSA. The protein solution was removed and 0.05% glutaraldehyde (100 µl) was subsequently added. After 45 min at room temperature, the glutaraldehyde was removed and the wells were washed twice with distilled water. BSA (100 µl of a 2% solution) was then added to the wells to block unoccupied sites and prevent non-specific binding of bacteria. After 30 min at room temperature, the BSA was removed and the wells were washed once with distilled water. Finally, the bacterial suspension (100 µl) was added and the plate was incubated at 37 °C for 1.5 h with gentle shaking every 20 min. After the attachment period, unbound bacteria were removed by washing the wells three times with PBS containing 0.01% Tween 20 to minimize non-specific hydrophobic interactions. Bacteria left adhering to the wells were fixed with Bouin's fixative (Ivey, 1980) for 10 min. After extensive washing with distilled water, bacteria were stained for 15 min with Hucker crystal violet (Sonnenwirth, 1980). Wells were then rinsed with distilled water and dried at 37 °C for 2 h. After adding 200 µl 95% (v/v) ethanol to each well, the plate was shaken vigorously to release the stain. The A_{590} was then recorded using an Easy Beam microtitre plate reader (SLT-Labinstruments). All assays were performed in triplicate and the means $\pm$ SD were calculated. In one experiment, the number of bound bacteria was estimated by measuring the A_{590} produced by stained bacterial films containing a known quantity of cells. In this case, the concentration of a cell suspension of *P. intermedia* was determined using a Petroff-Hausser counting chamber according to the manufacturer's instructions. Various dilutions of the suspension were added to the wells of a microtitre plate, the bacteria were dried, fixed and stained, and the A_{590} was measured, as described above.

Effect of pH on collagen-binding activity. The collagen-binding assay was performed using suspensions of *P. intermedia* BMH prepared in the following buffers at 50 mM: citrate buffer (pH 3, 4, 5), phosphate buffer (pH 6, 7), Tris/HCl buffer (pH 8, 9) and carbonate buffer (pH 10).

Effect of putative inhibitors on collagen-binding activity. Putative inhibitors of bacterial adherence were incorporated in the bacterial suspension, and the collagen-binding assay was carried out as described above. The following compounds were tested: human serum (1:10 dilution in PBS); stimulated whole human saliva (1:10 dilution in PBS); 50 mM EDTA; various carbohydrates at 100 mM (galactose, glucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine); various proteins at 0.1% (BSA, gelatin, IgG, fibrinogen, fibronectin, type I acid-soluble collagen, type IV acid-soluble collagen); various amino acid and synthetic peptides at 0.1% [glycine, proline, polyglycine (molecular mass = 2–5 kDa), polyproline (molecular mass = 1–10 kDa), poly(Pro-Gly-Pro) (molecular mass = 2–10 kDa), Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr (a fibronectin-related peptide)].

Effect of treatments on collagen-binding activity. Bacterial cells were incubated at 4 °C (1 month), 37 °C (2 d), 50 °C (30 min) or 60 °C (30 min) before the collagen-binding activity was assayed. The bacterial cells were also submitted to a light ultrasonic treatment in order to detach any loosely bound structures or proteins. In this case, the bacterial suspension was sonicated (3 $\times$ 10 s, 20% duty cycle; Sonic dismembrator model 150, Artek Systems) and the cells were washed twice by centrifugation before measuring collagen-binding activity. pH stability of the binding activity was tested by incubating bacteria

at room temperature for 60 min at either pH 2 or pH 12. The sensitivity of the collagen-binding activity to proteolytic enzymes was evaluated by incubating bacteria at 37 °C for 2 h in the presence of either trypsin or proteinase K (1 mg ml⁻¹). The bacterial cells were also treated at room temperature for 1 h with constant agitation in the presence of 100 mM EDTA, 400 mM sodium chloride, 100 mM sodium periodate, 0.01% 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS), 0.01% hexadecyltrimethylammonium bromide (HTAB), 0.01% SDS, 0.01% Triton X-100, 0.01% Tween 20 or 0.01% Zwittergent 3-14. All the above-treated bacteria were washed twice and suspended ($OD_{660} = 2.0$) in PBS before the collagen-binding activity was assayed.

RESULTS

All nine strains of *P. intermedia*/*P. nigrescens* tested in the study attached to various extents to type I collagen (calf skin) immobilized onto wells of a microtitre plate (Table 1). No attachment occurred when wells were coated with BSA. Coating the wells with type I human collagen resulted in an attachment comparable to that of type I calf-skin collagen (data not shown). Among the other bacterial species tested, only *Porphyromonas gingivalis* ATCC 33277 and *Eubacterium saburreum* 162.4 bound significantly to the collagen-coated wells (Table 1). In the former case, the adherence did not appear to be specific since bacteria also attached to the same extent to the control well covered with BSA. A second strain of *Por. gingivalis* (W83) did not bind to the collagen film. *P. intermedia* strain BMH was selected for further characterization of the collagen-binding activity.

Table 1. Ability of selected oral bacteria to attach to collagen immobilized onto wells of a microtitre plate

Adherence is measured as the A_{590} value.

Strain	Adherence (mean $\pm$ SD)
<i>Prevotella intermedia</i> BMH	0.67 $\pm$ 0.08
<i>Prevotella intermedia</i> BH 20/30	0.52 $\pm$ 0.10
<i>Prevotella intermedia</i> MS/5B/372	0.32 $\pm$ 0.09
<i>Prevotella intermedia</i> G8-9K-3	0.87 $\pm$ 0.13
<i>Prevotella nigrescens</i> T2	0.68 $\pm$ 0.11
<i>Prevotella nigrescens</i> S19g	0.40 $\pm$ 0.07
<i>Prevotella nigrescens</i> NCTC 9336	0.30 $\pm$ 0.08
<i>Prevotella nigrescens</i> Cg1265	0.23 $\pm$ 0.10
<i>Prevotella nigrescens</i> 5W2	0.37 $\pm$ 0.05
<i>Prevotella loeschii</i> ATCC 15930	0
<i>Porphyromonas gingivalis</i> ATCC 33277	0.81 $\pm$ 0.14
<i>Porphyromonas gingivalis</i> W83	0.09 $\pm$ 0.05
<i>Treponema denticola</i> ATCC 35405	0
<i>Actinobacillus actinomycetemcomitans</i> 702	0
<i>Eubacterium saburreum</i> 162.4	0.17 $\pm$ 0.08
<i>Actinomyces viscosus</i> 54.2	0
<i>Actinomyces naeslundii</i> 85.1	0
<i>Streptococcus mutans</i> ATCC 10449	0

Table 2. Effect of various putative inhibitors on adherence of *P. intermedia* BMH to collagen immobilized onto wells of a microtitre plate

Compound	Concentration*	Percentage adherence (mean $\pm$ SD)
None (control)		100
EDTA	50 mM	25 $\pm$ 11
Serum	Dilution 1:10	0
Saliva	Dilution 1:10	19 $\pm$ 9
BSA	0.2 %	104 $\pm$ 15
Gelatin	0.2 %	7 $\pm$ 6
Type I acid-soluble collagen	0.05 %	12 $\pm$ 7
Type IV acid-soluble collagen	0.05 %	12 $\pm$ 5
Fibrinogen	0.2 %	29 $\pm$ 13
Fibronectin	0.025 %	15 $\pm$ 5
IgG	0.2 %	97 $\pm$ 10
Galactose	100 mM	97 $\pm$ 7
N-Acetyl-D-galactosamine	100 mM	101 $\pm$ 7
Glucose	100 mM	109 $\pm$ 15
N-Acetyl-D-glucosamine	100 mM	104 $\pm$ 9
Glycine	0.2 %	103 $\pm$ 12
Proline	0.2 %	112 $\pm$ 17
Polyglycine	0.2 %	99 $\pm$ 6
Poly-L-proline	0.2 %	95 $\pm$ 7
Poly(Pro-Gly-Pro)	0.2 %	93 $\pm$ 10
Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr	0.2 %	90 $\pm$ 12

* Final concentration in the assay.

Preliminary experiments showed that the adherence of *P. intermedia* BMH to the collagen film was time- and bacterial-concentration-dependent (data not shown). The adherence assay was found to be reproducible; triplicate experiments gave A_{590} values ranging from 0.62 to 0.76 (0.67 ± 0.08). Using bacterial suspensions with known concentrations, it was estimated that in the assay performed, the number of cells attached to the collagen film was approximately 2×10^7 per well. Mid-exponential-phase cultures of *P. intermedia* demonstrated the greatest capacity to bind to the collagen film ($A_{590} = 0.85 \pm 0.1$). A significant decrease in attachment was observed with cells from a 6-d-old culture ($A_{590} = 0.32 \pm 0.07$). Optimal attachment occurred between pH 7 and pH 8, whereas no binding was observed when the assay was carried out at pH 3 or pH 10.

Table 2 presents data concerning the effect of various putative inhibitors on the adherence of *P. intermedia* to collagen-coated wells. Incorporation of the chelating agent EDTA in the bacterial suspension was associated with a 75 % reduction of bacterial attachment. Among the proteins tested, gelatin, type I and type IV collagens, fibrinogen, as well as fibronectin, reduced the attachment of *P. intermedia*. Human serum and saliva also interfered with the adherence, whereas no effect was observed with BSA and IgG. Carbohydrates, amino acids and peptides tested had no inhibitory effect. Synthetic peptides with a high content in glycine and proline (as for collagen), as

well as a fibronectin-related peptide, did not interfere with the binding.

The effect of various cell treatments on the collagen-binding activity of *P. intermedia* is reported in Table 3. The collagen-binding activity was not eliminated by a light ultrasonic treatment. The bacteria could be stored for up to one month at 4 °C or for 2 d at 37 °C with no apparent loss of binding activity. Collagen-binding activity was extinguished by treatment of the bacteria at 60 °C for 30 min, at pH 2 or 12, as well as by incubation in the presence of proteinase K or sodium periodate. The collagen-binding activity was not affected by treatment with trypsin. Among the various detergents used, Zwittergent 3-14 was the only one to have a significant effect; after treating cells with it, collagen-binding activity could not be demonstrated. Incorporating the resulting detergent fraction during the adherence assay could also prevent the binding of untreated cells (data not shown). No such inhibition was demonstrated with the other detergents used in the investigation.

DISCUSSION

The microtitre plate adherence assay used in this study was found to be convenient. It gives an excellent reproducibility, is easy to perform and does not require sophisticated equipment. Furthermore, a number of cell

Table 3. Effect of various treatments of *P. intermedia* BMH on adherence to collagen immobilized onto wells of a microtitre plate

Treatment	Percentage adherence (mean $\pm$ SD)
None	100
Light sonication	104 $\pm$ 10
Storage	
37 °C/2 d	92 $\pm$ 12
4 °C/1 month	99 $\pm$ 8
Heat	
50 °C/30 min	95 $\pm$ 11
60 °C/30 min	12 $\pm$ 4
pH*	
2	4 $\pm$ 3
12	7 $\pm$ 13
Proteolytic enzymes†	
Trypsin (1 mg ml ⁻¹)‡	96 $\pm$ 9
Proteinase K (1 mg ml ⁻¹)‡	5 $\pm$ 6
Chemicals*	
EDTA (100 mM)‡	80 $\pm$ 16
NaCl (400 mM)‡	95 $\pm$ 6
Sodium periodate (0.1 M)‡	3 $\pm$ 4
Detergents*	
CHAPS (0.01 %)‡	95 $\pm$ 7
HTAB (0.01 %)‡	83 $\pm$ 11
SDS (0.01 %)‡	80 $\pm$ 14
Triton X-100 (0.01 %)‡	76 $\pm$ 12
Tween 20 (0.01 %)‡	97 $\pm$ 8
Zwittergent 3-14 (0.01 %)‡	4 $\pm$ 6

* 25 °C for 1 h.

† 37 °C for 2 h.

‡ Final concentration during treatment.

treatments and putative inhibitors of adherence can be tested in a short period of time to characterize the nature of the binding component.

The data presented in this report suggest a specific interaction between *P. intermedia* and type I collagen. Non-specific binding of *P. intermedia* to collagen must be ruled out because (i) no attachment of bacteria to BSA-coated wells was detected and (ii) complete inhibition of attachment occurred in the presence of free soluble collagen or its denatured form (gelatin). Collagen-binding capacity has been previously demonstrated for other oral bacterial species, including certain streptococci of the mutans group (Liu & Gibbons, 1990), *Actinomyces viscosus* (Liu *et al.*, 1991) and *Por. gingivalis* (Naito & Gibbons, 1988). The collagen-binding activity of the mutans streptococci is heat-sensitive and not affected by carbohydrates, fibrinogen and fibronectin (Liu & Gibbons, 1990). *A. viscosus* attaches to human type I collagen but not to its denatured form or to type I rat collagen. It has

been suggested that this binding to collagen is associated with type 1 fimbriae rather than type 2 fimbriae (Liu *et al.*, 1991). The collagen-binding activity of *Por. gingivalis* is heat-sensitive and inhibited by human serum, IgG, fibrinogen and fibronectin (Naito & Gibbons, 1988).

The fact that incorporation, in the adherence assay, of fibronectin, a collagen-binding glycoprotein, prevents the attachment of *P. intermedia* suggests that the same binding site on the collagen molecule may be involved in the binding of fibronectin and bacteria. This is reinforced by the fact that preincubation of bacteria with fibronectin did not reduce their ability to adhere to collagen and that fibronectin-coated wells did not allow attachment of *P. intermedia* (data not shown).

On the basis of heat sensitivity and susceptibility to proteinase K, the receptor for collagen on *P. intermedia* may contain a proteinaceous moiety. Furthermore, the inhibition obtained by treating cells with sodium periodate suggests that the binding component may be a glycoconjugate. Devine & Handley (1989) have reported that a proteinase-K-sensitive, trypsin-resistant component on fibrils of *P. intermedia* is involved in the coaggregation reaction of the bacteria with *Actinomyces* spp. Based on these characteristics, the same receptor may be involved in the attachment of *P. intermedia* to *Actinomyces* spp. and to collagen.

The plasma protein fibrinogen could also prevent the attachment of *P. intermedia* to collagen-coated wells. Binding of fibrinogen by *P. intermedia* has been previously reported by Lantz *et al.* (1985). It was shown that this heat-sensitive adherence mechanism is not affected by the presence of EDTA. On the contrary, the collagen-binding activity reported in the present study was strongly inhibited by the chelating agent. It is possible then that inhibition by fibrinogen is related to steric hindrance, due to the close proximity of both receptors, rather than to a direct competition for the same binding component. One should not rule out the possibility that some fibronectin contamination present in the commercial fibrinogen preparation may be responsible for the inhibition. The finding with respect to the Zwittergent 3-14 extraction of the collagen-binding component may be used to develop a strategy for the purification of the putative ligand.

The present investigation reports for the first time the ability of *P. intermedia* to bind collagen. Since the major constituent of periodontal tissues is collagen, primarily type I in the tissue and type IV in the basement membrane (Narayanan & Page, 1983), it is suggested that the collagen-binding capacity of *P. intermedia* may play a key role in tissue invasiveness and colonization by providing a mechanism allowing the cells to attach to a tissue matrix.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada.

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Received 7 November 1995; revised 22 January 1996; accepted 24 January 1996.