Original

Effect of the Neuraminidase Purified from
Prevotella loescheii on Hemagglutination
by *Porphyromonas gingivalis*

Porphyromonas gingivalis の赤血球凝集反応に
及ぼす *Prevotella loescheii* より精製された
ノイラミニダーゼの影響

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概要: *Porphyromonas gingivalis* の赤血球凝集機構に関与するシアル酸の役割について追究するため, *Prevotella loescheii* より精製したノイラミニダーゼを用いて, *P. gingivalis* の赤血球凝集反応に及ぼす精製ノイラミニダーゼの影響について検討した。ヒトO型赤血球を精製酵素で処理して, マイクロタイタープレートを用いた連続2倍希釈法で *P. gingivalis* の赤血球凝集活性を測定したところ, アシアロ赤血球(血球表面の糖タンパク質よりシアル酸を取り除いた赤血球)にすると *P. gingivalis* の赤血球凝集能が増大した。次いで, 分光光度計による赤血球凝集活性測定法により, *P. gingivalis* 381株の赤血球凝集能に及ぼす種々の因子について追究した。*P. gingivalis* 381株菌体の赤血球凝集活性は, 正常赤血球, アシアロ赤血球いずれも, 供試した糖やアミノ酸では阻害を受けなかった。また, サルミン, cGMP 依存性キナーゼ阻害因子, アンジオテンシン I, II および III のようなアルギニンを含むペプチドでは 正常赤血球ならびにアシアロ赤血球に対して, 程度に差はあるが, 凝集の阻害がみられたが, サルミンは双方の赤血球に対し低濃度で凝集を特異的に阻害した。次いで, 正常赤血球表面よりシアル酸を精製酵素で除去した際, 赤血球表面の荷電状態の変化を調べるために, 正常赤血球およびアシアロ赤血球表面のゼータ電位を測定した。その結果, 正常赤血球のゼータ電位は $-18.3 \pm 3.10 \text{ mV}$ であったが, アシアロ赤血球のゼータ電位は $-4.4 \pm 1.16 \text{ mV}$ と著明に変動した。さらに, 酵素量を変化させて, 赤血球表面より段階的にシアル酸を除去したところ, 赤血球からの遊離シアル酸量が増大するにつれて, 赤血球表面のゼータ電位は0値に近づき, かつ, *P. gingivalis* 381株菌体の赤血球凝集活性は増大する傾向を示した。以上の結果より, 本菌とアシアロ赤血球との凝集能が, 正常赤血球と比べて増大する現象には, 赤血球表面のゼータ電位の低下が密接に関与していることが明らかにな

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Introduction

Sialic acid is a component of carbohydrate chains in various glycoproteins such as salivary glycoproteins, serum glycoproteins, and glycoproteins of cell membranes. In studies on salivary-bacterial interactions^{1,2)}, the removal of sialic acid residues of various salivary glycoproteins, such as mucin and other bacterial agglutinins, diminished their interactions with *Streptococcus sanguis* and *Streptococcus mitis*. In contrast, the abilities of *Actinomyces* strains to cause hemagglutination and to bind to epithelial cells were enhanced by removing sialic acid from erythrocytes (RBCs)^{3,4)} and epithelial cells⁵⁾. Thus, the presence or absence of sialic acid residues in glycoproteins appears to influence interactions between sialoglycoproteins and bacteria in the oral cavity. *Porphyromonas gingivalis*, which has been demonstrated to increase in subgingival dental plaque samples of human periodontal lesions⁶⁾, can agglutinate RBCs and bind to sulcular epithelium and saliva-coated hydroxyapatite⁷⁾. However, it has not been determined whether sialic acid residues in glycoconjugates are involved in interactions between *P. gingivalis* and host surfaces.

Neuraminidase (EC 3.2.1.18) cleaves α -ketosidic linkages between *N*-acetylneuraminic acid (NANA) and the remaining carbohydrate chains of oligosaccharides, glycolipids and glycoproteins. Activity of neuraminidase in the oral cavity has been found in dental plaque⁸⁾, in salivary secretions⁹⁾, and in crevicular fluid¹⁰⁾. Several investigators demonstrated that neuraminidase was present in the culture supernatant of some oral streptococci^{11,12)}. Previously we sought the presence of neuraminidase in culture supernatant and cell sediment from 29 strains of oral anaerobic bacteria which are believed to be intimately related to periodontal disease. Strong activity of neuraminidase was found in the culture medium of *Prevotella loescheii*¹³⁾, and the neuraminidase was purified¹⁴⁾. In this study, we examined the effect of purified neuraminidase-treatment of RBCs on hemagglutination by *P. gingivalis*.

Materials and Methods

Bacterial strains and growth conditions

The strains of black-pigmented anaerobic rods used in this study were obtained from the Research Laboratory of Oral Biology, Sunstar Inc., Osaka, Japan. All strains were maintained on plates containing Trypticase soy agar (BBL Microbiology System, Cockeysville, MD) supplemented with 5 % sheep blood; yeast extract (BBL), 1 mg/ml; hemin, 5 μ g/ml; and menadione, 1 μ g/ml. The strains of black-pigmented anaerobic rods were grown in 100 ml of Trypticase soy broth (BBL) supplemented with yeast extract, 1 mg/ml; hemin 5 μ g/ml; menadione 1 μ g/ml. Cultures were incubated in an Anaerobic System 1024 (Forma, Marietta, OH) in an atmosphere of N_2 - H_2 - CO_2 (80 : 10 : 10).

Purification of neuraminidase

The neuraminidase was purified from the culture medium of *P. loescheii* ATCC 15930, as described previously¹⁴⁾.

Hemagglutination assay

The cells of black-pigmented anaerobic rods used here were harvested from the culture, washed three times by centrifugation at $10,000 \times g$ for 20 min in 10 mM phosphate-0.15 M NaCl, pH 7.0 (PBS), and suspended in the same buffer to an optical density of 1.0 at 660 nm. Human O-type RBCs were washed three times by centrifugation at $1,000 \times g$ for 5 min in PBS and were used within 2 days of collection.

Neuraminidase-treatment of washed RBCs was performed by incubation with 10 ml of 10 % (vol/vol) packed-cell suspension in 10 mM acetate-0.15 M NaCl buffer, pH 5.5 and 0.5 unit of the purified neuraminidase for 60 min at 37°C. One unit of enzyme was defined as the amount which releases 1 μ mol of NANA per min, as measured by Uchida *et al.*^{15).}

A visual assay was performed at room temperature with a microtiter plate (Linbro Division Flow Laboratories Inc., Hamden, CONN). Serial dilutions of bacterial cell suspensions were mixed with equal volumes of 2% RBC suspensions. The mixtures were left to stand for 60 min, and hemagglutination was determined by observing the mixture without magnification. Titer was expressed as the reciprocal of the highest dilution showing positive agglutination.

Hemagglutination of *P. gingivalis* was also followed by a turbidimetric method. The progress of hemagglutination was monitored by the absorbance at 542 nm (A_{542}) for 15 min at 37°C using a UV-visible recording spectrophotometer and a temperature-controlled cuvette compartment with a magnetic stirrer (UV-265 FW, Shimadzu). Suspensions of about 5×10^7 of RBCs and about 3×10^8 bacterial cells were mixed with 10 mM PBS (pH 7.0) or inhibitor to a total volume of 2 ml in a cuvette. The hemagglutinating activity was defined as the difference in optical density at 542 nm between 0 time and 10 min after starting the reaction.

Determination of zeta potential

The method for preparation of RBC and bacterial suspensions was the same as that described for the turbidimetric method of hemagglutination. The zeta potentials were determined from electrophoretic mobilities by means of a Lazer-zee meter Model 501 (Pen-Kem, Inc., Bedford Hills, NY), which automatically incorporates the conductivity of the suspension and is based on the application of the Smoluchowski equation^{16).} The voltage used was 100 V. The electrophoretic mobility of each sample was measured 10 times at 37°C.

Results

The hemagglutinating titers of black-pigmented anaerobic rods using neuraminidase-treated and untreated RBCs are shown in Table 1. *P. gingivalis* strains tested had strong hemagglutinating activity

Table 1 Hemagglutinating titer of black-pigmented anaerobic rods for the purified neuraminidase-treated and untreated RBCs

Species and strain		Hemagglutinating titer for*	
		Native RBCs	Neuraminidase-treated RBCs
<i>P. gingivalis</i>	381	128	512
	Sun-3	128	512
	ATCC 33277	256	1024
	I-2-1	256	1024
<i>P. intermedia</i>	ESS-1	2	8
	ATCC 25261	2	16
<i>P. denticola</i>	ATCC 33185	2	8
<i>B. levii</i>	14015	0	0
	JP-2-77	0	0
<i>P. loescheii</i>	ATCC 15930	0	0

* Titer was expressed as the reciprocal of the highest dilution showing positive agglutination on a microtiter plate.

Fig. 1 Hemagglutination reactions with *P. gingivalis* cells and either the native RBCs (A) or neuraminidase-treated RBCs (B) by continuous recording of turbidity measurements.

after the start of the reaction than that of untreated RBCs.

The effect of various compounds on hemagglutination of neuraminidase-treated and untreated RBCs by *P. gingivalis* 381 was further studied by the turbidimetric method. The hemagglutination of both RBCs was unaffected by 5 mM sugars or amino acids such as D-galactose, D-mannose, L-fucose, *N*-acetylglucosamine, *N*-acetylglactosamine, NANA, glycine, L-alanine, L-valine, L-serine, L-arginine, L-lysine, L-proline, and L-leucine (data not shown). The results of hemagglutination-inhibition by some peptides are shown in Table 2. All arginine-containing peptides tested inhibited the hemagglutination of untreated RBCs. The inhibitory effect of these peptides except salmine was reduced by neuraminidase-treatment of

and the strains of *Prevotella intermedia* and *Prevotella denticola* showed weak activity. The ability of these organisms to hemagglutinate was strongly enhanced by neuraminidase-treatment of the RBCs. However, strains of *Bacteroides levii* and *P. loescheii* agglutinated neither neuraminidase-treated nor untreated RBCs.

The representative curves shown in Fig. 1 are actual recorder tracings for the hemagglutination reactions of the neuraminidase-treated and untreated RBCs by cells of *P. gingivalis* 381 according to a turbidimetric method. The hemagglutination of enzyme-treated RBCs caused a more rapid and greater decrease of the A_{542}

Table 2 Effect of neuraminidase-treatment of RBCs on hemagglutination inhibition with peptides

Peptide	Amino acid sequence	Relative hemagglutinating activity (%)	
		Native RBCs	Neuraminidase-treated RBCs
Control		100	100
Salmine(0.01mM)	Pro-Arg-Arg-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-Arg-Arg-Arg-Pro-Arg-Val-Ser-Arg-Arg-Arg-Arg-Arg-Gly-Gly-Arg-Arg-Arg	0	0
cGMP dependent inhibitor(0.1mM)	Arg-Lys-Arg-Ala-Arg-Lys-Glu	2	25
Angiotensin I (0.1mM)	Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu	3	95
Angiotensin II (0.1mM)	Asp-Arg-Val-Try-Ile-His-Pro-Phe	42	97
Angiotensin III (0.1mM)	Arg-Val-Try-Ile-His-Pro-Phe	16	68
Bradykinin-potentiator C (0.1mM)	Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	100	100

*The reaction mixture that did not contain the peptide was used as a control. The relative hemagglutinating activity was calculated on the basis of control by a turbidimetric assay.

the RBCs. Bradykinin potentiator C, which has no arginine residue, did not have an inhibitory effect.

For the native RBCs and *P. gingivalis*, zeta-potentials were -18.3 ± 3.10 mV and -9.80 ± 1.30 mV (mean $\pm$ S.D.), respectively. The changes in hemagglutinating activity of *P. gingivalis* 381, zeta potential of RBCs and amount of NANA released during the course of neuraminidase-treatment are shown in Fig. 2. With increasing amounts of NANA released from the RBCs by neuraminidase, the zeta potential of the RBCs approached zero and hemagglutination yielded higher activity.

Discussion

Studies on hemagglutination of human RBC by strains of *P. gingivalis* may contribute to knowledge about the adherence of these bacteria to oral mucosal surfaces and possibly to the glycoprotein pellicle of teeth. In general, the mechanism of hemagglutination by bacteria involves specific and/or non-specific interactions between RBCs and bacteria. Oral bacteria such as *A. viscosus*, *A. naeslundii*^{3,4)} and *Eikenella corrodens*¹⁷⁾ were found to have lectin-like proteins on their surfaces which bind to D-galactose or N-acetylgalactosamine-like receptors on the RBC surface, and their hemagglutination was enhanced by neuraminidase removal of terminal NANA. However, hemagglutination of both neuraminidase-treated and untreated RBCs by *P. gingivalis* was not inhibited by sugars including D-galactose and N-acetylgalactosamine (see the Results). Thus, bacterial lectins may not be involved in hemagglutination of *P. gingivalis*. Similar results were reported by Inoshita *et al.*¹⁸⁾, Grenier *et al.*¹⁹⁾ and Okuda *et al.*²⁰⁾. They also showed that hemagglutination of *P. gingivalis* was inhibited by amino acids including L-arginine and L-lysine. However, in this study, hemagglutination was inhibited by arginine-containing peptides (Table 2) but not by the free amino acids tested (see the Results). Among arginine-containing peptides, salmine, which has many arginine residues, was the most potent inhibitor. Glycophorin A, a major sialoglycoprotein of the RBC membrane, has a polypeptide chain containing arginine residues²¹⁾. Thus, it is hypothesized that hemagglutination by *P. gingivalis* would involve ligand-binding with arginine-containing peptides as receptors on the RBC surface.

Using the neuraminidase-treated RBCs, arginine-containing peptides, except salmine, were found to be less inhibitory for hemagglutination by *P. gingivalis*. Although it is difficult to explain these findings, it is feasible that neuraminidase removal of NANA would influence non-specific forces including electrostatic and hydrophobic interactions. When the hydrophobicity of neuraminidase-treated and untreated RBCs was measured by hexadecane-adsorption test, no difference was found between them (data not shown). However, neuraminidase gradually increased agglutinability of RBCs as it reduced zeta-potential in proportion to the amount of NANA removed. Pollack²²⁾ proposed that the natural cohesive forces between RBCs in electrolyte solutions were opposed by the net negative surface charge of the cells. This net negative surface charge is largely due to the ionized carboxyl groups of NANA¹⁶⁾. The zeta-potential is the potential difference between the cell surface and the kinetically bound cloud of ions surrounding the RBC.

Fig. 2 Effect of release of NANA from RBCs by the neuraminidase on zeta potential of RBCs and hemagglutinating activity of *P. gingivalis* 381.

With a decrease in zeta-potential, the thickness of the double-layer consisting of the negatively charged RBC as one layer and the diffuse cloud of cations as the other also may decrease. Therefore, the enhancing effect of neuraminidase could be explained as a result of the decrease in surface charge and, thus, zeta-potential.

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