Evidence for independent molecular identity and functional interaction of the haemagglutinin and cysteine proteinase (gingivain) of *Porphyromonas gingivalis*

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Summary. The sequence of events involved in haemagglutination and lysis of erythrocytes by washed cells, vesicles and the culture supernate of *Porphyromonas gingivalis* strain W83 was monitored by ^{51}Cr release and transmission electronmicroscopy. All preparations, except capsular material and lipopolysaccharide, caused haemagglutination and, by a slow process of attachment and specific attack on the surface structures of the red blood cells, produced minute pores and eventual leakage of cellular contents. N-acetylglucosamine, N-acetylgalactosamine and several other sugars such as glucose and sucrose had no effect on haemagglutination. Antiserum raised against a cloned haemagglutinin of *P. gingivalis* strain 381 inhibited the activity of strain W83 cells, vesicles and supernate. The antiserum-neutralised supernate lost 70–80% of its hydrolytic activity towards α -N-benzoyl-L-arginine-4-nitroanilide but the residual activity behaved in a manner similar to the native supernate in that it was completely inhibited by the addition of 2,2'-dipyridyl disulphide and was fully restored upon addition of a low- M_r mercaptan. Binding of the antiserum to the haemagglutinin epitope of *P. gingivalis* still permitted titration of the active centre cysteinyl thiol group of the proteinase. Purified gingivain caused lysis of erythrocytes and was not neutralised by antiserum to the haemagglutinin. These results suggest that, although the haemagglutinin and gingivain are probably separate molecules, they are closely associated on the outer membrane of *P. gingivalis* and may be functionally related.

Introduction

Porphyromonas gingivalis is unique among the genera *Porphyromonas* and *Prevotella* in possessing both a haemagglutinin and a potent membrane-bound proteinase. Both are regarded as potential virulence determinants for *P. gingivalis*; consequently their nature and function are currently under intensive investigations in several laboratories.

Many of the characteristics of the haemagglutinin of *P. gingivalis* have been reported^{1–6} but the nature of the molecule remains elusive. The haemagglutinin was first reported by Okuda and Takazoe⁷ and subsequently shown to comprise two major proteins of 40 and 60 Kda.⁶ Inoshita *et al.*³ purified a haemagglutinin from the culture supernate of strain 381 and identified three major proteins (24, 37 and 44 Kda). Two polypeptides of 43 and 49 Kda, associated with the haemagglutinating activity of strain

ATCC 33277, were also reported¹ and antiserum to the haemagglutinin was demonstrated in the sera of healthy individuals and patients with chronic periodontitis.² Recently, at least one haemagglutinin gene has been cloned in *Escherichia coli*^{8, 9} and antisera to the clone reacted with a 43 Kda protein of the cell lysates of *P. gingivalis* strains 381 and ATCC 33277. Polyclonal antiserum to the haemagglutinin of strain 381 (expressed in recombinant *E. coli* clone ST2) was previously shown to react mainly with the 43- and 38-Kda peptides and to a lesser extent with other peptides (30, 32, 105 and 115 Kda) in the cell lysate of its corresponding strain.⁹ Hybridisation studies have indicated that the cloned 381 haemagglutinin gene is present also in the other human isolates of *P. gingivalis* tested including strains W83 and W50 (Lepine and Progulske-Fox, unpublished observation). Furthermore, immuno-electronmicroscopy techniques revealed that the haemagglutinin resides on the cell surface of *P. gingivalis*.⁹ Its location was also confirmed indirectly by the use of a pleiotropic mutant of strain W50 (W50/BE1) which had lost its haemagglutinating activity,¹⁰ probably due to the absence of its outer-membrane proteins.¹¹

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The nature of the proteinase of *P. gingivalis* is more controversial. The enzyme has been referred to simply as a protease, a serine protease, a thiol protease and most frequently as a trypsin-like enzyme.¹²⁻¹⁷ The use of the latter designation has led to a widespread use of trypsin inhibitors in experiments designed to determine the function of this enzyme. Recently, we have purified the extracellular proteinase of *P. gingivalis* and presented evidence that the enzyme is not trypsin-like but is a cysteine proteinase for which we proposed the name "gingivain".¹⁸ We have shown previously that gingivain causes lysis of erythrocytes.⁶ It has been suggested that the haemagglutinin and the protease of *P. gingivalis* are the same molecule.⁴⁻⁶ In the present investigation, we extend our observations to elucidate the sequence of events that lead to the process of haemolysis. A series of experiments with monospecific antiserum to the cloned haemagglutinin of *P. gingivalis* and a thiol-specific inhibitor of gingivain, 2,2'-dipyridyl disulphide, was designed to study the interaction of these two processes.

Materials and methods

Growth conditions and vesicle preparation

P. gingivalis strain W83 was grown in BM liquid medium containing haemin and menadione as described previously¹⁹ at 37°C in an atmosphere of N₂ 70%, H₂ 20%, CO₂ 10%. The culture supernate and cells after growth for 6 days were separated by centrifugation at 18 000 *g* for 30 min. Vesicle preparations were obtained from the culture supernate by 70% (w/v) followed by 40% (w/v) ammonium sulphate fractionation as described previously.⁶

Capsular material

Capsular material was extracted as described previously.²⁰ Briefly, 500 mg (wet weight) of cells were suspended in 100 ml of NaCl (0.89%) solution, and mixed for 6 h at 4°C. Two equivalent volumes of chilled acetone were added and the mixture was centrifuged at 20 000 *g* for 1 h at 8°C. The acetone layer was collected and concentrated under reduced pressure. The resulting precipitate was dialysed against double distilled water for 2 days and then dried under vacuum. Capsular material was further purified by column chromatography with Sepharose 4B (Pharmacia, Uppsala, Sweden) and 50 mM, Tris-HCl, pH 8.0, as eluant. The eluate was monitored continuously for total carbohydrate content as described by Dubois *et al.*²¹ A single major peak (c. 70% carbohydrate, 5% protein) was recovered and designated the capsular material. Protein concentrations were determined by the method of Lowry *et al.*²²

Cysteine proteinase (gingivain)

The cysteine proteinase was prepared from the culture supernate, as described previously,⁶ by am-

monium sulphate fractionation (70% saturated) and covalent chromatography^{23, 24} with activated thiopropyl-Sepharose 6B (Pharmacia). The enzyme was eluted by a concentration gradient of 10–20 mM cysteine in a buffer containing 300 mM NaCl and 300 mM Tris-HCl, pH 8.0. Residual cysteine in the enzyme preparation was removed by gel filtration (Sephadex G25; Pharmacia) with 0.1 M KCl as the eluant.

Lipopolysaccharide (LPS)

LPS was extracted from 1 g wet weight of *P. gingivalis* strain W83 cells by the method of Westphal and Jann.²⁵ It was further purified by sequential treatment with DNAase, RNAase and pronase, followed by ultracentrifugation at 100 000 *g* for 1 h as described previously.²⁶ The crude LPS preparation was further extracted with phenol-chloroform-petroleum ether to remove traces of macromolecular contaminants as described by Galanos *et al.*²⁷ The purified LPS was electrodialed²⁸ to remove low-M_r contaminants such as amines.

Haemagglutination and haemolysis

Haemagglutination was assayed in microtitration plates by mixing fresh sheep red blood cells 0.5% with serial two-fold dilutions of 50 µl of test components in a total volume of 250 µl. *P. gingivalis* washed cells, vesicle preparation, culture supernate, LPS and capsular material were tested. Titres were reported as the endpoint dilution/ml of assay mixture. The experiments were repeated with ⁵¹Cr-labelled erythrocytes. Briefly, red blood cells were incubated in phosphate-buffered saline containing 12.5 µCi of chromium for 2 h, after which, cells were repeatedly washed in buffer-saline until no radioactivity was measured in the washes. Inhibition of haemagglutination was measured under the above conditions except that the compounds tested were added serially, in two-fold dilutions, to the concentration of the highest dilution that caused haemagglutination. Carbohydrate solutions (200 mM) tested for their inhibitory effects were D-glucose, D-galactose, D-fructose, D-mannose, D-sucrose, D-N-acetylglucosamine and D-N-acetylgalactosamine. Neutralisation with antiserum to the *P. gingivalis* haemagglutinin (ST2) was tested as above. The effect of the thiol-specific inhibitor 2,2'-dipyridyl disulphide (2PDS) was also tested at a pre-determined concentration that was just sufficient to cause complete inhibition in the assays with α-N-benzoyl-L-arginine-4-nitroanilide (BAPNA; Sigma) as substrate. The effect of the removal of 2PDS by a low-M_r mercaptan on the haemagglutination inhibition was examined by incorporating 5 µl of 20 mM cysteine. Similarly, a mixture of 5 µl of the cysteine solution and antiserum (titre of 1600) was also tested. Lysis of erythrocytes was assessed by the release of ⁵¹Cr and electron-microscopy. Samples for electronmicroscopy were negatively stained with phosphotungstic acid, pH 6.5,

2% and examined in a Phillips 400 transmission electronmicroscope as described previously.¹⁹

Determination of catalytic activity

Catalytic activity of the culture supernate and gingivain towards BAPNA was determined at 25°C, in 100 mM Tris-HCl buffer, pH 7.4, by initial rate analysis in a continuous spectrophotometric assay at 412 nm with a Gilford model 240 spectrophotometer, essentially as described by Malthouse and Brocklehurst.²⁹ The stock solution of BAPNA (1.5 mM) was made up in water containing dimethyl sulphoxide 1% v/v. A typical assay mixture contained 800 µl of 0.1 mM Tris-HCl buffer and 100 µl of enzyme solution. The reaction was started by adding 100 µl of BAPNA solution and the increase in A_{412} was recorded. Rates were calculated from the linear traces and converted into units of $M^{-1}s^{-1}$ by $\epsilon_{412} = 8.8 \times 10^3 M^{-1} cm^{-1}$ as described by Erlanger *et al.*³⁰

Reactions with 2,2'-dipyridyl disulphide (2PDS)

2 PDS (Aldrich) was recrystallised twice from light petroleum (b.p. 60–80°C) and had a m.p. of 58°C. Inhibition experiments were performed by adding 2PDS solution (50 µl of 1.48 mM) to assay mixtures in which catalytic activity had been demonstrated, and reactivation was achieved subsequently by adding 5 µl of 20 mM cysteine solution. The cysteine residues in gingivain or the supernate were titrated by allowing 2PDS to react with each preparation at pH 4 and pH 8 and determining the increase in A_{343} .

Results

Washed cells, supernate and vesicle preparations of *P. gingivalis* grown in BM liquid medium for 6 days resulted in haemagglutination (1060, 40510 and 87620 haemagglutination units/ml, respectively). Haemagglutination was not affected by hexoses (e.g., glucose, galactose and fructose), N-acetylglucosamine or N-acetylgalactosamine in accord with the recent findings of Kay *et al.*³¹ for *P. gingivalis* strain W50. The above experiments were repeated with red blood cells labelled with ^{51}Cr ; release of ^{51}Cr was detected after exposure of the red blood cells to *P. gingivalis* components for 2 h and increased steadily up to 4 h, after which no further changes were observed (fig. 1). ^{51}Cr release was accompanied by peculiar morphological distortions on the surface of the red blood cells as revealed by electronmicroscopy (fig. 2a). After incubation for 2 h, the erythrocytes ruptured at some of these distorted sites (fig. 2b), followed by leakage of cell contents and, after 4 h, only the membranes of the erythrocytes were visible. In wells containing gingivain and red blood cells only, direct lysis was clearly evident. It was possible to differentiate readily between lysis which

Fig. 1. ^{51}Cr release from sheep erythrocytes after incubation: (a) with gingivain; (b) following haemagglutination.

occurred in <2 h and haemagglutination followed by lysis which became apparent after 4 h.

Capsular material did not agglutinate erythrocytes, nor did LPS purified by enzymic treatment, ultracentrifugation and electrodialysis at concentrations up to 10 µg/ml.

Antiserum against the cloned haemagglutinin of *P. gingivalis* strain 381⁹ inhibited the haemagglutinating activity of *P. gingivalis* strain W83 cells, vesicles and supernate, whereas haemolysis due to gingivain remained unaffected. The hydrolytic activity of the supernate on BAPNA was completely inhibited by 2PDS, but was reactivated by addition of a low- M_r mercaptan such as cysteine (20 mM) (fig. 3). However, the antibody-neutralised supernate (titre 1600) retained only 20–30% of its initial activity towards BAPNA (fig. 3). Similarly, this residual activity was completely inhibited by 2PDS and reactivated by the addition of cysteine (fig. 3). Spectrophotometric titration specifically of the active site cysteinyl thiol-group is achieved by reaction with 2PDS at pH 4.0 (see Brocklehurst³² for a description of the basis of this technique). At pH 8.0, the absorbance change at 343 nm was approximately two-fold greater than at pH 4.0 (fig. 4) which resulted from the additional reaction of a non-functional cysteinyl thiol group. These results are in accord with those obtained previously for purified gingivain.⁶

The addition of 2PDS inhibited the cysteine proteinase gingivain in the supernate, preventing both haemagglutination and cell lysis. This inhibitory effect was reversed by the addition of 20 mM cysteine and after incubation for 4 h, cell lysis was apparent. When a mixture of cysteine and antiserum was added, neither haemagglutination or cell lysis were apparent.

Discussion

The haemagglutinating activity of *P. gingivalis* has been reported by several workers.^{1–6} Recently, we

Fig. 2. Stages in the lysis of erythrocytes after treatment with *P. gingivalis* cells, vesicles or supernate. (a) Red blood cells are grossly distorted with pronounced protrusions, but still intact, within 1 h ($\times 8\ 400$). (b) Leakage of erythrocyte contents through minute pores, after 2 h ($\times 22\ 400$). (c) Ghosting of red blood cells as a consequence of steps (a) and (b) resulting eventually in disintegration of the membranes after 4 h ($\times 8\ 400$).

have shown that haemagglutination is accompanied by lysis.⁶ In this study, the sequence of events leading to haemagglutination and lysis was followed over a period of 5 h. Haemagglutination was not accompanied by simultaneous lysis, but rather a slow process of attachment and specific attack on the red-blood-cell surface components, leading to the production of minute pores and leakage of cellular contents of the erythrocytes.

P. gingivalis possesses surface components such as a capsule, fimbriae, LPS and outer-membrane proteins, some of which have been implicated in haemagglutination.^{1, 2, 5, 31} In the present study, highly purified capsular material and LPS showed no haemagglutinating activity. Similarly, fimbriae of *P. gingivalis* have previously been shown to lack haemagglutinating properties. Growing evidence implicates a protein-lipid complex in the outer membrane in the mediation

Fig. 3. Inhibition and reactivation of *P. gingivalis* supernate (S) and supernate plus antiserum (SA). (a) The supernate or the supernate plus antiserum was continuously monitored at 412 nm until the baseline was unchanged. (b, b') The addition of BAPNA to the assay mixture leads to an increase in absorbance at 412 nm due to the chromophore *p*-nitroaniline. (c, c') Inhibition of hydrolytic activity towards BAPNA by the addition of stoichiometric amounts of 2PDS to the reaction mixture. (d, d') The addition of a low-M_r mercaptan (SH) such as cysteine resulted in reactivation of hydrolytic activity towards BAPNA.

Fig. 4. Reaction of the thiol groups of gingivain with 2PDS to produce the chromophoric product, pyridine-2-thione ($\epsilon_{343} = 8080 \text{ M}^{-1} \text{ s}^{-1}$); (a) at pH 4.0, only the catalytically essential thiol group reacts rapidly; (b) at pH 8.0, both the catalytically non-functional thiol group and the catalytically essential thiol group react rapidly.

of haemagglutination.^{5,7} Recent findings by Nishikata *et al.*⁴ showed, from proteinase-inhibitor studies, that the haemagglutinin of *P. gingivalis* possesses proteolytic properties. This was supported by our previous finding that haemagglutination was inhibited by the thiol-blocking agent 2PDS.⁶ These results suggest that haemagglutination and proteolytic activity are due to a surface molecule on *P. gingivalis* cells, or vesicles secreted into the culture medium. Such a hypothesis requires that the haemagglutinin and the cysteine proteinase are situated in the outer membrane and extend beyond its surface to enable effective binding and proteolysis to occur.

The current study suggests that a haemagglutinin and proteinase may exist as separate but juxtaposed structural entities on the outer membrane, but may be functionally interdependent. Thus, when an excess of antiserum is added to the reaction mixture, 20–30% of the hydrolytic activity towards BAPNA remains (fig. 3); furthermore, the catalytic thiol group of the proteinase is still accessible to the 2PDS inhibitor (fig. 4). These results, together with the observations that purified gingivain does not cause haemagglutination, suggest that the antibody binds a neighbouring region of the outer membrane close to the enzyme surface. Binding of antibody close to the enzyme could reduce its catalytic activity by distortion of its conformation or by direct interference with substrate binding. The abolition of haemagglutination by the reaction of membrane-bound gingivain with 2PDS suggests that a part of the enzyme surface close to the catalytic site and the binding area of the enzyme might be involved in binding of antibody and haemagglutination. Derivatisation of the thiol group as a bulky 2-pyridyl

disulphide might result in steric shielding of the postulated adjacent part of the enzyme surface.

P. gingivalis has an obligatory requirement for haem, and its capacity to grow on blood-agar plates and hydrolyse haemoglobin indicate that these micro-organisms can obtain haem directly from whole blood. Furthermore, *P. gingivalis* demetallates protohaem to produce protoporphyrin, which can be stored and utilised by the organism. This may be an important mechanism for the acquisition of iron, which is essential for the growth of most bacteria.³³ *P. gingivalis* has a requirement for iron in the ferrous form,³⁴ but the oxidation state of iron in the porphyrin molecule of this species is not yet known. Physiological concentrations of free iron are well below that required by micro-organisms; hence the capacity to demetallate protohaem under conditions of haem excess may serve to redress this imbalance.

Haem has been shown both to increase the mean generation time and modulate the virulence of *P. gingivalis*, and pleiotropic haem-deficient mutants have reduced virulence in animals.³⁵ The present results suggest that haemagglutination, followed by lysis of erythrocytes, may serve as a means of obtaining both haem and iron *in vivo*. Other members of the genus *Porphyromonas* lack the ability to either haem-agglutinate or lyse erythrocytes, and this deficiency might partly explain their lower pathogenic potential. Free haemoglobin has been shown to increase the virulence of many opportunistic pathogens.³³ However, haemoglobin is rapidly bound to haptoglobin *in vivo* and, therefore, is not freely available in serum (and presumably in crevicular fluid). *P. gingivalis* has been shown to degrade several iron binding proteins, including haptoglobin.³⁶ Thus further studies are required to determine whether this species can degrade haemoglobin-haptoglobin complexes.

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