The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50

Philip D. Marsh, Ann S. McDermid, Ailsa S. McKee
and Arthur Baskerville

Author for correspondence: Philip D. Marsh. Tel: +44 980 612287. Fax: +44 980 612731.

Pathology Division, PHLS
Centre for Applied
Microbiology and Research,
Porton Down, Salisbury
SP4 0JG, UK

***Porphyromonas gingivalis* strain W50 was grown under haemin-limitation and haemin-excess conditions in a chemostat at pH 7.5. The maximum specific growth rate ($\mu_{\max}$) was determined at both haemin concentrations ($\mu_{\max} = 0.236 \pm 0.052$ and 0.271 ± 0.039 h⁻¹, respectively). This enabled dilution rates to be adjusted so that the virulence and enzyme activity of haemin-limited and haemin-replete cells could be compared at identical relative growth rates (μ_{rel}) of 0.25, 0.50 and 0.75 of their respective $\mu_{\max}$. The data showed that the fastest growing cells were significantly more virulent than those grown more slowly, irrespective of haemin concentration. However, at each growth rate tested, cells grown under haemin-excess conditions were always more virulent than haemin-limited cells. Trypsin-like enzyme activity of whole cultures was also greater at each growth rate under haemin-excess conditions while, conversely, collagenolytic activity was generally higher in haemin-limited cultures. Thus, although growth rate had an effect on the virulence and enzyme activity of *P. gingivalis*, the availability of haemin for growth was the most significant factor.**

Keywords: *Porphyromonas gingivalis*, growth rate, haemin, virulence, proteolytic activity

INTRODUCTION

Porphyromonas (formerly *Bacteroides*) *gingivalis* is an obligately anaerobic, Gram-negative bacillus that has been implicated in the pathogenesis of advanced periodontal disease in humans (Mayrand & Holt, 1988). This species is also highly proteolytic and collagenolytic, and is virulent in experimental animal models, causing spreading, inflammatory infections when mono-infected in rodents (van Steenberg *et al.*, 1982).

P. gingivalis requires haemin for growth (Gibbons & MacDonald, 1960). It probably satisfies this requirement *in vivo* by degrading haem-containing and haem-complexing host proteins such as haptoglobin, haemopexin and albumin (Beaven *et al.*, 1974; Carlsson *et al.*, 1984), or haemoglobin derived from lysed red blood cells in the gingival crevice (Mukherjee, 1985). At times, this species may be growing in the host under haemin limitation since outer-membrane proteins, expressed only by *P. gingivalis* when grown *in vitro* under haemin limitation, were recognized by sera from patients with severe progressive periodontal disease (Papaioannou *et*

al., 1991). At other times, there may be an excess of this essential cofactor during periods of bleeding during disease activity (Mukherjee, 1985).

In an earlier study, cells of *P. gingivalis* that were grown at a fixed growth rate under haemin limitation were found to be less virulent than cells grown at the same growth rate under haemin excess (McKee *et al.*, 1986). The explanation for this was proposed to be due, in part, to the production of lower levels of a trypsin-like protease under haemin limitation (Marsh *et al.*, 1988), which is considered an important virulence factor for this organism (Slots & Genco, 1984). Subsequently, however, it was reported that changes in growth rate affected the production of hydrolytic enzymes by *P. gingivalis* (Minhas & Greenman, 1989). These authors argued that as we had not necessarily compared cells grown at the same proportion of their maximum growth rate (i.e. at the same relative growth rate, μ_{rel} ; Evans, 1976; Tempest, 1976), the difference in virulence we had observed could be due to a growth-rate effect rather than a haemin-effect on hydrolytic enzyme activity. Consequently, we have compared the virulence

of *P. gingivalis* grown under both haemin limitation and haemin excess at the same relative growth rates.

METHODS

Bacterial strains and growth conditions. *Porphyromonas gingivalis* strain W50 was grown in an LH 500 series II chemostat with a 500 ml working volume, at 37 °C, pH 7.5 ± 0.2, in an atmosphere of 5% CO₂/95% N₂ (v/v). The medium was BM (Shah *et al.*, 1976), modified as described previously (McKee *et al.*, 1986) to give conditions of haemin limitation (0.05 mg l⁻¹) or haemin excess (2.5 g l⁻¹). The amount of haemin needed to be added to BM in order to achieve haemin limitation was lower than that reported in our previous studies (McKee *et al.*, 1986; Marsh *et al.*, 1988). This was probably due to higher levels of endogenous haemin in the particular batches of media. Peptides were presumed to be the limiting nutrients during growth under haemin excess (McKee *et al.*, 1986). The maximum specific growth rate was determined under haemin limitation and haemin excess conditions by the wash-out method of Tempest (1970). The dilution rate used for wash-out was 0.5 h⁻¹. For subsequent experiments, the dilution rate was adjusted to grow the organism under haemin limitation or haemin excess at the same relative growth rates ($\mu_{rel} = \mu/\mu_{max}$; Evans, 1976; Tempest, 1976) of 0.25, 0.50 and 0.75 of their respective μ_{max} . The cell yields were determined by viable counts on horse blood agar (McKee *et al.*, 1986) during steady-state growth at each relative growth rate. Chemostats were set up fresh for each growth rate; ten pot volume changes of medium were allowed to elapse before a culture was considered to have reached a steady-state.

Virulence test. The virulence of cells grown in the chemostat under haemin limitation and haemin excess at the same relative growth rate was compared in mice as described previously (McKee *et al.*, 1986). Briefly, cells taken directly from the chemostat were concentrated by centrifugation (10000 g for 10 min) and suspended in fresh BM medium. The number of viable cells present was determined by plating decimal dilutions of this concentrated fraction on horse blood agar plates, and performing colony counts after incubation for 14 d at 37 °C in an atmosphere of 10% CO₂/10% H₂/80% N₂ (v/v). Female Porton strain albino mice were anaesthetized with ether and injected subcutaneously with 0.1 ml of each bacterial suspension. Deaths were recorded daily for up to 14 d after infection; surviving and moribund animals were killed with ether at the end of the study.

Enzyme assays. At each steady-state, cultures taken on different days directly from the chemostat were tested for trypsin-like protease activity (EC 3.4.21.4) and for the breakdown of collagen. Trypsin-like activity was determined in a recording spectrophotometer from the rate of hydrolysis of 1.0 mM *N*- α -benzoyl-L-arginine-*p*-nitroanilide (BAPNA, Sigma) at 405 nm following the addition of 0.1 ml of culture. Substrate was dissolved daily in 0.05 M phosphate buffer, pH 7.5. One unit of activity (U) caused a change in absorbance of 0.001 min⁻¹; specific activity was expressed as U per 10⁸ viable cells.

The assay for collagen breakdown was based on that of Mandl *et al.* (1953), in which helical regions of the molecule are degraded liberating peptides which are estimated spectrophotometrically as leucine. Culture samples (0.1 ml) were incubated anaerobically for 18 h with 25 mg of type 1 insoluble collagen from bovine achilles tendon (Sigma) that had previously been pre-treated with pepsin to remove contaminating proteins and denatured collagen (McDermid *et al.*, 1988). The collagen was suspended in 5 ml 0.05 M phosphate buffer, pH 7.5. After incubation, duplicate samples (0.25 ml) were mixed with 1 ml of

freshly prepared ninhydrin reagent, and heated for 20 min in a boiling water bath. When the mixture was cool, 5 ml propan-1-ol (BDH)/water (1:1, v/v) was added; after 15 min, the absorbance at 600 nm was measured and compared with that of a leucine standard; standard collagenase from *Clostridium histolyticum* (Sigma) was used as a positive control, and heat-killed cells served as a negative control. One unit of activity (U) liberated 1 μ mol leucine from collagen in 18 h at 37 °C; specific activity was expressed as U per 10⁸ viable cells.

Statistical analysis. In order to analyse the data on the virulence of *P. gingivalis*, the results were pooled and the differences between cells grown under haemin limitation and haemin excess, and among cells grown at different growth rates (μ_{rel}), were compared using the large sample proportions test (Fisher & van Belle, 1993).

Enzyme data for the haemin-limited/excess conditions and at the three growth rates were compared by two-way analysis of variance using the Statgraphics computer package (STSC Inc., Rockville, MD, USA).

RESULTS

Growth characteristics of *P. gingivalis*

The maximum specific growth rates (μ_{max}) under conditions of haemin limitation and haemin excess were 0.236 ± 0.052 ($n = 5$) and 0.271 ± 0.039 ($n = 5$) h⁻¹, respectively. For subsequent experiments, cells were grown under haemin limitation at dilution rates of 0.06, 0.12 and 0.18 h⁻¹, and under haemin excess at dilution rates of 0.07, 0.14 and 0.20 h⁻¹, to give μ_{rel} values of 0.25, 0.50 and 0.75 μ_{max} , respectively, for both haemin-limited and haemin-excess conditions.

Virulence of *P. gingivalis*

At each growth rate tested, cells of *P. gingivalis* that were grown under haemin-excess conditions were more virulent than their haemin-limited counterparts. There was also a growth rate effect, in that cells grown at the fastest rate (0.75 μ_{max}) were more virulent than cells grown under the same haemin concentration at slower rates (Table 1). The most virulent cells were those growing under haemin excess at the fastest growth rate.

Since the range of infective doses were comparable under all growth conditions, it was considered appropriate to pool all the mouse virulence data to enable statistical analysis of the differences observed between cells grown under haemin limitation and haemin excess (irrespective of growth rate), and among cells grown at different relative growth rates (irrespective of haemin concentration). Using the large sample proportions test, cells grown under haemin excess were significantly more virulent in mice than those grown haemin-limited ($\chi^2 = 6.48$, $P < 0.001$). Cells grown at 0.75 μ_{max} also caused a significantly greater proportion of deaths than those grown at either 0.50 or 0.25 μ_{max} ($\chi^2 = 3.83$ and 5.18, respectively; $P < 0.001$ in both cases). The difference in proportions of mice killed by cells grown at 0.25 and 0.50 μ_{max} was not statistically significant ($\chi^2 = 1.33$, $P = 0.18$).

Table 1. Effect of relative growth rate (μ_{rel}) and haemin concentration on the virulence of *P. gingivalis*

Virulence is expressed as the number of mouse deaths/total number of mice challenged. Statistical analysis of the data is described in the text.

μ_{rel}	Haemin limited		Haemin excess	
	Subcutaneous infective dose ($\log_{10}$ c.f.u.)	Virulence	Subcutaneous infective dose ($\log_{10}$ c.f.u.)	Virulence
0.25	9.44	0/10	9.40	7/10
	9.13	1/10	9.11	4/10
			8.75	2/10
0.50	10.49	5/10	9.69	6/10
	10.19	0/10	9.40	4/10
	9.89	1/10	9.09	8/10
0.75	9.60	4/10	10.03	10/10
	9.30	4/10	10.08	10/10
			9.38	9/10

Table 2. Effect of relative growth rate (μ_{rel}) on trypsin-like activity and collagenolytic activity of *P. gingivalis*

Activities are expressed as U per 10^8 viable cells $\pm$ SD. Numbers in parentheses represent the number of determinations. Statistical analysis of the data is described in the text.

μ_{rel}	Haemin limitation		Haemin excess	
	Trypsin-like activity	Collagenolytic activity	Trypsin-like activity	Collagenolytic activity
0.25	39.0 $\pm$ 4.5 (6)	1.1 $\pm$ 0.5 (10)	52.8 $\pm$ 10.0 (8)	1.5 $\pm$ 1.2 (10)
0.50	39.5 $\pm$ 5.9 (6)	21.5 $\pm$ 5.3 (10)	48.8 $\pm$ 1.3 (5)	3.4 $\pm$ 0.8 (10)
0.75	34.2 $\pm$ 15.7 (8)	16.3 $\pm$ 7.3 (10)	89.8 $\pm$ 26.8 (7)	4.5 $\pm$ 2.9 (10)

Enzyme activities

The enzyme activities of cultures taken during steady-state growth at each growth rate are listed in Table 2. No attempt was made to distinguish between cell-bound and extracellular activities. At each growth rate, haemin-excess cultures had greater trypsin-like activity than those grown haemin-limited. Growth rate had no effect on the trypsin-like activity of haemin-limited cultures, whereas the fastest growing culture had the highest trypsin-like activity during growth under haemin excess. In contrast, haemin-limited cultures displayed higher collagenolytic activity at 0.5 and 0.75 μ_{max} .

Two-way analysis of variance (ANOVA) was performed on the enzyme data in order to compare the effects of both growth rate and of haemin concentration. This analysis showed that haemin-excess conditions were associated with significantly higher levels of trypsin-like activity ($P \ll 0.001$, $F_{1,34} = 32.50$). Trypsin-like activity also varied significantly with different growth rates ($0.001 < P < 0.005$, $F_{2,34} = 6.47$). There was also a significant

interaction effect between growth rate and haemin concentration ($P < 0.001$, $F_{2,34} = 10.92$). The major source of this effect was the higher trypsin-like levels detected under haemin excess at $\mu_{rel} = 0.75$ (Table 2).

Increased collagenolytic activity was highly significantly associated with haemin-limited conditions ($P \ll 0.001$, $F_{1,55} = 93.85$). Higher growth rates were also significantly associated with greater levels of collagenolytic activity ($P \ll 0.001$, $F_{2,55} = 45.67$), with the most significant effect due to the difference between the lowest growth rate (0.25 μ_{max}) and the higher growth rates (0.5 and 0.75 μ_{max}) under haemin limitation. There was also a significant interactive effect on collagenolytic activity of growth rate and haemin limitation ($P \ll 0.001$, $F_{2,55} = 30.85$).

DISCUSSION

It is well established that the growth environment can alter the pathogenicity of bacteria (e.g. Smith, 1980; Taylor, 1984). This study confirms and extends previous

research which showed that the concentration of haemin in the growth environment can affect the virulence of *P. gingivalis* (McKee *et al.*, 1986). In our earlier work it was found that cells grown with an excess of haemin were more virulent than a comparable infective dose of cells grown at the same fixed growth rate under haemin limitation (McKee *et al.*, 1986). Subsequent work by Minhas & Greenman (1989), however, showed that hydrolytic enzyme production, including the trypsin-like protease, varied with the growth rate of *P. gingivalis*. Since we had compared the virulence of haemin-limited and haemin-excess cells grown at the same fixed growth rate and not at the same relative growth rate, Minhas & Greenman (1989) proposed that the difference in virulence we observed might be due to a growth-rate- rather than to a haemin-effect on the physiology of cells. Consequently, in the present study, we compared the virulence and proteolytic activity of *P. gingivalis* grown under haemin limitation and haemin excess at the same physiological pace (i.e. at the same μ_{rel}). Indeed, as predicted by Minhas & Greenman (1989), a significant growth-rate-effect on virulence was observed, with the fastest growing cells being more virulent than those grown at slower rates, irrespective of the haemin concentration of the medium. At each growth rate tested, however, haemin-excess cells were always more virulent than their haemin-limited counterparts, and the significance of the haemin effect was stronger than that associated with growth rate, confirming the significance of haemin to the pathogenic potential of this organism.

The higher trypsin-like and lower collagenolytic activities of haemin-excess cultures were in agreement with earlier findings obtained during growth at a single, fixed growth rate (Marsh *et al.*, 1988). Although collagen breakdown is important for tissue destruction (McKee *et al.*, 1986), the data from this study and from earlier work suggest that high levels of trypsin-like activity might determine whether infection or disease occurs.

The precise role played by haemin in the virulence of *P. gingivalis* is not understood fully at present. Cultures grown under haemin excess display greater proteolytic (e.g. the trypsin-like protease) activity, and produce higher concentrations of metabolic end-products such as ammonia, propionate and butyrate (McKee *et al.*, 1986; Marsh *et al.*, 1988) that possess cytotoxic activity (Touw *et al.*, 1982). It has also been shown recently, however, that cells (including outer membranes and extracellular vesicles) grown under haemin excess bind more haemin than do their counterparts grown under haemin limitation (Smalley *et al.*, 1991). Thus, the enhanced virulence of cells grown under haemin excess may be due, in part, to their ability to initiate growth *in vivo* by utilizing cell-bound haemin prior to cells being able to acquire peptides and haemin directly from the degradation of haem-containing host molecules.

In conclusion, this study has demonstrated that both growth rate and haemin concentration can have a significant effect on the virulence and enzyme activity of *P. gingivalis*, although the influence of haemin was

stronger. At each growth rate tested, cells grown in an excess of haemin were always more virulent than those grown at a comparable rate under haemin limitation.

ACKNOWLEDGEMENT

The authors thank Dr D. J. Bradshaw for his help with the statistical analysis of the data.

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Received 2 August 1993; revised 17 October 1993; accepted 17 November 1993.