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Hemin nutritional stress inhibits bacterial invasion of radicular dentine by two endodontic anaerobes

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Abstract

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Aim To determine if anaerobic bacteria routinely found in infected dentine and root canals require the presence of heme in the environment in order for them to invade dentinal tubules.

Methodology Noncarious, unrestored human teeth with single root canals were prepared for invasion experiments and soaked in either TSB-M supplemented with hemin (5 μg mL^{-1}) ($n = 12$ roots), TSB-M media ($n = 12$ roots) or TSB-M media followed by hemin soak ($n = 12$ roots) for 2 days, then inoculated with either *Prevotella intermedia* ATCC 25611 or *Peptostreptococcus micros* ATCC 33270 and incubated anaerobically for 14 days. Roots were prepared for light microscopy, stained with Brown and Brenn or antisera raised to the bacteria, and invasion within tubules assessed using a tubule invasion index (TI). Data were analysed using Student's *t*-test and Mann–Whitney *U*-test.

Results *Prevotella intermedia* (TI = 0.7 ± 0.04) and *P. micros* (TI = 0.96 ± 0.08) showed low invasion when grown in the presence of hemin with cells generally restricted to the superficial 20 μm of the tubules, whilst neither bacteria invaded tubules (TI = 0) when hemin was absent from the growth media ($P < 0.01$).

Conclusions Hemin was required in the growth medium for *P. intermedia* and *P. micros* to invade dentinal tubules.

Keywords: anaerobe, dentine, hemin, invasion, nutrition, stress.

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Introduction

Bacteria-induced pulp and periapical disease is associated with invasion and colonization of the pulpodentine complex by oral microorganisms. Much is known on the composition, formation and development of dental plaque, however, relatively little is known about the mechanisms by which bacteria invade dentine. An understanding of these mechanisms may allow the development of control strategies in prophylactic, restorative, or endodontic treatment. Studies have demonstrated that tubule invasion is associated with specific bacterial cell wall–polypeptide interactions with unmineralized collagen (Love *et al.* 1997) and other bacteria (Love *et al.* 2000), and bacterial interactions with various molecules found in tissue fluid, dentinal fluid and saliva (Love 2001, 2002). The presence of an appropriate nutritional supply in the environment influences bacterial growth (Loesche *et al.* 1983, ter Steeg *et al.* 1987) and may influence disease progression. Studies examining the bacterial flora of carious coronal dentine show that facultative species that primarily obtain energy from fermenting carbohydrates, for example *Streptococcus* spp., are mainly located in superficial dentine whilst fastidious obligate anaerobic bacteria that can metabolize host-derived amino acids and peptides, such as *Peptostreptococcus micros* and *Prevotella intermedia*, are located in deeper dentine (Edwardsson 1987, Ozaki *et al.* 1994). The micoflora of noncarious dentine probably follows a similar pattern (Love & Jenkinson 2002) whilst the microflora within radicular dentine of teeth with

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infected root canals resembles that of deep layers of carious dentine (Ando & Hoshino 1990, Peters *et al.* 2001). These studies demonstrate that the environment deep within the dentine favours obligate anaerobes and this may be due to low oxygen tension and the supply of nutrients in the tubule derived from tissue fluid, bacterial bi-products and disintegrated pulp and dentinal tubule constituents.

Heme (iron protoporphyrin) is an important growth factor for many oral obligate anaerobes and this has to be acquired from the host (Carlsson *et al.* 1984) as these bacteria generally lack the ability to synthesize it (Gibbons & MacDonald 1960). It is likely that a source of heme would be present within dentinal tubules either in its free form when released from damaged erythrocytes or bound to heme-sequestering proteins, e.g. albumin. This study was conducted to determine if *P. micros* and *P. intermedia*, bacteria routinely found in infected dentine (Edwardsson 1987, Peters *et al.* 2001) and root canals (Sundqvist 1992), require the presence of heme in the environment in order for them to invade dentinal tubules.

Materials and methods

Bacterial strains and culture media

Cells of *P. micros* ATCC 33270 or *P. intermedia* ATCC 25611 were grown anaerobically (85% N_2, 10% H_2 and 5% CO_2, 37 °C) on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with hemin (5 μg mL^{-1}), menadione (0.5 mg mL^{-1}), and (5% v/v) sheep whole blood (sTSB agar). Frozen (−80 °C) stocks were made by suspending cells in reduced Trypticase soy broth (BBL Microbiology Systems) containing 1 mg mL^{-1} yeast extract (Difco Laboratories, Detroit, MI, USA), 1 μg mL^{-1} menadione (TSB-M), supplemented with 5 μg mL^{-1} hemin, and glycerol (15% v/v).

Bacterial invasion of dentinal tubules

To ensure that bacterial penetration of dentine occurred by bacterial growth and invasion rather than by nonspecific means, noncarious, unrestored human teeth with single root canals were prepared for invasion experiments as described elsewhere (Love *et al.* 1997), with slight modification. Briefly, immediately following extraction of the teeth, they were cleaned with periodontal curettes and 5.25% (w/v) sodium hypochlorite (NaOCl) to remove adherent nondental hard and soft tissues, with care being taken not to damage the cementum. The crowns were then removed at the cemento-enamel junction using a rotating diamond saw (Isomet Saw, Buhler Ltd, Evanston, IL, USA) with water irrigation. The roots were inspected for root fractures, lateral canals or resorptive defects with a stereomicroscope at 12× magnification and were discarded if defects were present. Canal patency was confirmed by passing a number 15 K-file through the apex and the canals were instrumented sequentially using ProFile rotary instruments (Dentsply Maillefer, Ballaigues, Switzerland) with copious irrigation of 17% (w/v) ethylenediaminetetraacetic acid (EDTA) and 5.25% (w/v) NaOCl to 1 mm from the apical foramen to a final size 20, .04 taper. The roots were then sectioned longitudinally through the root canal with the diamond saw to produce two specimens. To ensure complete removal of the smear layers the prepared roots were placed in an ultrasonic bath (L&R T-14; L&R Manufacturing Co., Kearny, NJ, USA) with 17% (w/v) EDTA for 4 min followed by 5.25% (w/v) NaOCl for 4 min. To remove any residual chemicals the root specimens were immersed in sterile water and subjected to pressure in a denture pressure flask for 10 min, and then stored in 1 L of sterile water for 1 week.

Thirty-six root specimens were selected at random, sterilized by autoclave for 30 min at 120 °C and organized into three groups and treated as follows:

Hemin-replet (control) groups: (i) six root specimens were submerged in 10 mL TSB-M supplemented with hemin (5 μg mL^{-1}) and soaked for 2 days under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2, 37 °C). Glycerol stock (10 μL mL^{-1}) of *P. intermedia* was introduced to the media and the suspension was incubated anaerobically for 14 days; (ii) six root specimens were similarly treated and *P. micros* (10 μL mL^{-1}) was introduced to the media and the suspension was incubated anaerobically for 14 days.

Hemin-deficient groups: (i) six root specimens were submerged in 10 mL TSB-M and soaked for 2 days under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2, 37 °C). Glycerol stock (10 μL mL^{-1}) of *P. intermedia* was introduced to the media and the suspension was incubated anaerobically for 14 days; (ii) six root specimens were similarly treated and *P. micros* (10 μL mL^{-1}) was introduced to the media and the suspension was incubated anaerobically for 14 days.

Hemin-soak groups: (i) six root specimens were submerged in 10 mL TSB-M supplemented with hemin (20 μg mL^{-1}) and soaked for 2 days under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2, 37 °C). The

growth media was removed and replaced with hemin solution (20 μg mL^{-1}) for 1 day. The hemin solution was then replaced with 10 mL TSB-M. Glycerol stock (10 μL mL^{-1}) of *P. intermedia* was introduced to the media and the suspension was incubated anaerobically for 14 days; (ii) six root specimens were similarly treated and *P. micros* (10 μL mL^{-1}) was introduced to the media and the suspension was incubated anaerobically for 14 days.

At 3-day interval media was removed from all groups, short of exposing the root specimens, and replaced with fresh media. To check for purity and viability of the cultures portions were plated onto sTSB agar and incubated as above. Optical density (OD_{600}) readings were taken daily to monitor bacterial growth.

Light microscopy

Preparation of root specimens

Infected root specimens were fixed in 10% (w/v) formalin buffered at pH 7, demineralized in 10% (w/v) formic acid containing 2% (w/v) formalin, neutralized in lithium carbonate, washed, dehydrated and blocked in wax. Transverse sections (6 μm) were cut from the root dentine at the cervical area.

Preparation of antisera

Antisera against whole cells of *P. micros* ATCC 33270 or *P. intermedia* ATCC 25611 were raised in male New Zealand white rabbits. Cells were fixed with 0.25% (v/v) glutaraldehyde in PBS (0.1 mol L^{-1} KH_2PO_4 KOH^{-1}, pH 7, containing 0.15 mol L^{-1} NaCl) at room temperature for 40 min. They were then diluted to 10 mL with PBS, washed three times with PBS, and suspended in PBS (4 mL). Portions (500 μL, containing approximately 1×10^9 cells) were emulsified with equal volume of Freund's incomplete adjuvant (500 μL) and injected subcutaneously on three separate occasions, 3–4 weeks apart. Antisera against each bacteria was raised in two rabbits. Trial blood samples were obtained and antibody titres were detected by ELISA, when a satisfactory antibody titre was obtained the rabbit was bled and the antisera was sterilized by filtration.

Detection of bacteria

Ten sets of two transverse root sections, taken 10 sections apart, were picked up onto gelatine-coated slides. The first section of each set was stained with Brown and Brenn stain (Brown & Brenn 1931). The second section was prepared for immunohistochemistry. Slides were heated at 60 °C for 30 min and at 37 °C for 16 h, placed in xylene (twice for 10 min), absolute ethanol (twice for 1 min), 2% H_2O_2 in 95% (w/v) methanol (10 min), and finally rinsed in water. Nonspecific binding sites were blocked by incubating sections with 2% (w/v) bovine serum albumin (BSA) in Tris-buffered saline [TBS; 50 mmol L^{-1} Tris–HCl (pH 7.5) containing 0.15 mol L^{-1} NaCl] at 20 °C for 5 min. Sections were incubated with antibodies diluted 1 : 1000 with TBS–BSA for 30 min, washed with TBS (twice for 15 min) and incubated with peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako Corporation, Carpinteria, CA, USA) diluted 1 : 30 in TBS–BSA for 30 min. Slides were washed, developed with 3,3′-diaminobenzidine, washed, and counter-stained with haematoxylin. Negative controls (no primary antibodies) were included in every immunohistochemistry run.

The extent of invasion was expressed as the tubule invasion index (TI) (Love *et al.* 1997). Briefly, the centre of the root canal surface was identified and from this position the canal wall was divided into six equal portions. The extent of bacterial invasion within each of the portions was recorded at ×400 magnification by determining the numbers of tubules containing bacteria. Where 1–20 tubules were infected, invasion was scored as 1, where 21–50 tubules were infected invasion was scored as 2, and >50 tubules infected was scored as 3. To calculate the TI the scores were added and the mean was designated the TI. A TI of <0.5 was classified as nil invasion, 0.5–0.99 as low invasion, 1.0–1.49 as mild, 1.5–1.99 as mild to moderate, 2.0–2.5 as moderate to heavy and >2.5 as heavy. Data were analysed using Student's *t*-test and Mann–Whitney *U*-test.

Results

For all experimental groups bacterial growth occurred and cells remained viable and as a pure culture over the 14-day experimental period. Growth in hemin-deficient media was slower for both species, however, OD_{600} readings from all groups became similar as the time period progressed. Brown and Brenn and immunohistochemically stained sections from the hemin-replete group revealed that cells of *P. intermedia* and *P. micros* demonstrated a similar invasion pattern characterized as low with cells generally restricted to the superficial 20 μm of the tubules (Fig. 1, Table 1). For the hemin-deficient group invasion was significantly different ($P < 0.01$) with neither species demonstrating bacterial invasion (Fig. 1, Table 1). The addition of hemin to the

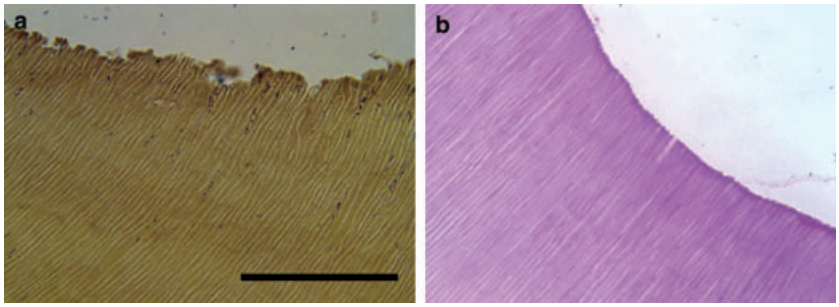

Figure 1 Representative sections demonstrating (a) low and superficial tubule invasion by *Peptostreptococcus micros* grown in a hemin-replete environment, and (b) nil invasion when grown in a hemin-deficient environment (Brown and Brenn stain, bar 20 μm).

Table 1 The effect of hemin on dentinal tubule invasion by *Prevotella intermedia* and *Peptostreptococcus micros*

	Tubule invasion index	
Group[a]	Brown and Brenn	Antisera
Hemin-replete		
P. intermedia	0.7 ± 0.04	0.66 ± 0.05
P. micros	0.96 ± 0.08	0.83 ± 0.08
Hemin-deficient		
P. intermedia	0	0
P. micros	0	0
Hemin-soak		
P. intermedia	0	0
P. micros	0	0

[a]$n = 6$ roots per experiment.

dentine (hemin-soak group) did not significantly ($P > 0.05$) enhance bacterial invasion by either species in hemin-deficient growth media (Table 1).

Discussion

The exact role of an individual bacterial species in a dental infection is difficult to determine because most infections are polymicrobial. Both *P. intermedia* and *P. micros* are obligate anaerobes implicated in periodontal disease and are commonly found within the anaerobic environment in dentine and the root canal (Edwardsson 1987, Sundqvist 1992, Peters *et al.* 2001). Although their role in endodontic infection is not fully understood it is likely that their virulence factors, e.g. attachment to host cells (Dzink *et al.* 1989) and protease production (Sundqvist 1993) are directed to damage to the periradicular tissues. Studies of mechanisms that allow these cells to invade dentine and root canal systems may allow the development of control strategies that could limit the development of periapical inflammatory disease.

A number of factors may influence tubule invasion such as cell interactions and nutritional requirements. A main determinant is tubule patency and this variable must be controlled when studying the ability of bacteria to invade tubules. This study only recorded the invasion pattern in the cervical radicular dentine as it has been shown that this area has a higher number and degree of patent tubules compared with the apical region (Nalbandian *et al.* 1960) that readily allows invasion by bacteria (Love 1996). Removing cementum enhances bacterial penetration, however, in an experimental model this may not reflect the invasion capability of cells as they may penetrate the tubules by nonspecific means such as diffusion, therefore cementum was left intact in this study. As a result the model used in this study maximized the ability to investigate the ability of bacteria to invade dentine by growth mechanisms.

Detection of bacterial species within dentinal tubules by light microscopy techniques can give a good indication of the invasion pattern with respect to the depth of invasion and the extent of infection within areas of dentine by recording the number of tubules infected. However, visualization and detection of Gram-negative cells such as *P. intermedia* may be difficult due to uptake of the stain and could result in lower invasion values or false negative results. To maximize the ability to detect *P. intermedia* immunohistochemical staining to raised antisera was used in this study and demonstrated that recording of invasion was similar to Brown and Brenn stain (Table 1) indicating the viability of the method and accuracy of the TI results.

Limiting nutritional supply within a dentinal tubule may influence the ability of bacteria to invade and the

depth of penetration. This is partly dependent upon the patency of the tubule as diffusion of substances into radicular tubules from the periradicular tissues is proportional to tubule diameter. Also, the anaerobic environment and presence of tissue components, e.g. hemin within tubules is likely to favour growth and survival of organisms such as *P. intermedia* and *P. micros*. The experiments were designed to maximize conditions for anaerobic growth within dentinal tubules and the results demonstrated that both *P. intermedia* and *P. micros* were able to invade dentinal tubules when grown in the presence of hemin, the invasion pattern was low and limited to the superficial portion of the tubule. This is consistent with an *ex vivo* study that reported limited shallow invasion by *P. intermedia* (Berkiten *et al.* 2000) and clinical studies demonstrating low numbers of *P. intermedia* and *P. micros* within dentinal tubules (Ozaki *et al.* 1994, Peters *et al.* 2001). When hemin was not present within the growth media, both species were able to grow, albeit at a reduced rate compared with growth in hemin; however, neither species was able to invade dentinal tubules. This may have been due to growth rate, however, even with a reduced growth rate it would be expected that some cells would have been detected within tubules. The results suggest that the ability to invade tubules is determined by the presence of an optimal environment and that nutritionally stressed *P. intermedia* and *P. micros* cells lose the ability to invade. Bacterial attachment to dentine (Hubble *et al.* 2003) and tubule invasion (Love *et al.* 1997, Love 2001) requires cells to express adhesins and it is known that stressed cells produce a different protein profile, directed to cell survival, than cells growing in an ideal environment (Hartke *et al.* 1998). This may account for the inability of the stressed *P. intermedia* and *P. micros* cells to invade dentine.

The hemin-soak experiment was an attempt to simulate a hemin-replete environment within dentinal tubules that may stimulate stressed (hemin-deplete) cells to invade, however no invasion by either species occurred. It is not clear what the mechanism behind this was but may be due to an inability of the stressed cells to utilize the substrate or the deposition of hemin in tubules physically impeded cell invasion (Love 2002).

Conclusion

A number of factors are related to bacterial invasion of dentinal tubules, the results show that *P. intermedia* and *P. micros* cells grown in optimal conditions possess the ability to invade dentinal tubules, whilst cells grown in a hemin-deficient environment lost their ability to invade. Under the limitations of the study it can be seen that cells grown under a stressed environment diminishes their ability to invade.

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