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INVASION OF DENTINAL TUBULES BY ORAL BACTERIA

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ABSTRACT: Bacterial invasion of dentinal tubules commonly occurs when dentin is exposed following a breach in the integrity of the overlying enamel or cementum. Bacterial products diffuse through the dentinal tubule toward the pulp and evoke inflammatory changes in the pulpo-dentin complex. These may eliminate the bacterial insult and block the route of infection. Unchecked, invasion results in pulpitis and pulp necrosis, infection of the root canal system, and periapical disease. While several hundred bacterial species are known to inhabit the oral cavity, a relatively small and select group of bacteria is involved in the invasion of dentinal tubules and subsequent infection of the root canal space. Gram-positive organisms dominate the tubule microflora in both carious and non-carious dentin. The relatively high numbers of obligate anaerobes present—such as *Eubacterium* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Peptostreptococcus micros*, and *Veillonella* spp.—suggest that the environment favors growth of these bacteria. Gram-negative obligate anaerobic rods, e.g., *Porphyromonas* spp., are less frequently recovered. Streptococci are among the most commonly identified bacteria that invade dentin. Recent evidence suggests that streptococci may recognize components present within dentinal tubules, such as collagen type I, which stimulate bacterial adhesion and intra-tubular growth. Specific interactions of other oral bacteria with invading streptococci may then facilitate the invasion of dentin by select bacterial groupings. An understanding of the mechanisms involved in dentinal tubule invasion by bacteria should allow for the development of new control strategies, such as inhibitory compounds incorporated into oral health care products or dental materials, which would assist in the practice of endodontics.

Key words. Dentinal tubule, endodontic infections, oral bacterial adhesion, caries, invasion of dentin.

(I) Introduction

Endodontics is the clinical discipline that deals with the prevention and management of diseases of the pulp and periapical tissues. Normally, the dental pulp (Fig. 1) is sterile and is primarily involved in the production of dentin and in tooth sensibility. The pulp and dentin form a functional complex that is protected from exogenous substances in the oral cavity by the overlying enamel or cementum. When the pulpo-dentin complex becomes infected (Fig. 1A), the tissues react to the invading bacteria in an attempt to eradicate them. The ability of the complex to perform this function should not be underestimated, since the tissues are richly endowed with immunocompetent processes. However, in clinical terms, if the route of infection is not eradicated by these natural processes, or by operative procedures, then the burden of bacteria invading the complex overcomes the defenses and causes pulp disease, e.g., pulpitis, necrosis, and infection of the pulp chamber and root canal.

The root canal space is in open communication with the periapical tissues (periodontal ligament, cementum, and alveolar bone) *via* the apical foramen (Fig. 1). Bacterial metabolites and toxic products arising from bacteria present within the root canal diffuse into the periapical tissues and evoke inflammatory disease, e.g., apical periodontitis, which is characterized by resorption of alveolar bone (Fig. 1B), while localized areas of root resorption may also occur. In situations where the periodontal ligament has been damaged, e.g., after dental trauma, an infected root canal can induce extensive and rapid

inflammatory root resorption. Bacterially induced periapical disease usually begins as a chronic inflammation and manifests histologically as a periapical granuloma. An acute apical periodontitis of endodontic origin indicates that the host defenses are unable to control the bacterial insult. This may be due to bacteria becoming established within the periapical tissues, with subsequent abscess formation, or due to the presence of specific bacteria within the root canal that are able to induce tissue destruction. The bacterial toxins and acute inflammatory response characteristically cause swelling and pain. The main goal of endodontic treatment is to eliminate bacteria from the root canal system and to prevent them from infecting or re-infecting the pulp, root canal, or periapical tissues. Successful treatment depends upon a sound understanding of the causative factors of the disease process.

Miller (1890) first demonstrated the bacterial invasion of dentinal tubules of both carious and non-carious dentin and reported that the tubule microflora consisted of cocci and rods. It was not until the late 1950s that experimental evidence clearly established the fundamental role of bacteria in dental caries and in pulp and periapical disease. Keyes (1960) was able to show that dental caries did not develop in germ-free animals fed a range of diets. Later, Kakehashi *et al.* (1965) demonstrated that pulp and periapical disease occurred in surgically exposed rat molar pulp only when bacteria were present in the oral cavity. In gnotobiotic (germ-free) rats, exposed pulps remained healthy and initiated repair by way of dentin bridging of the exposure.

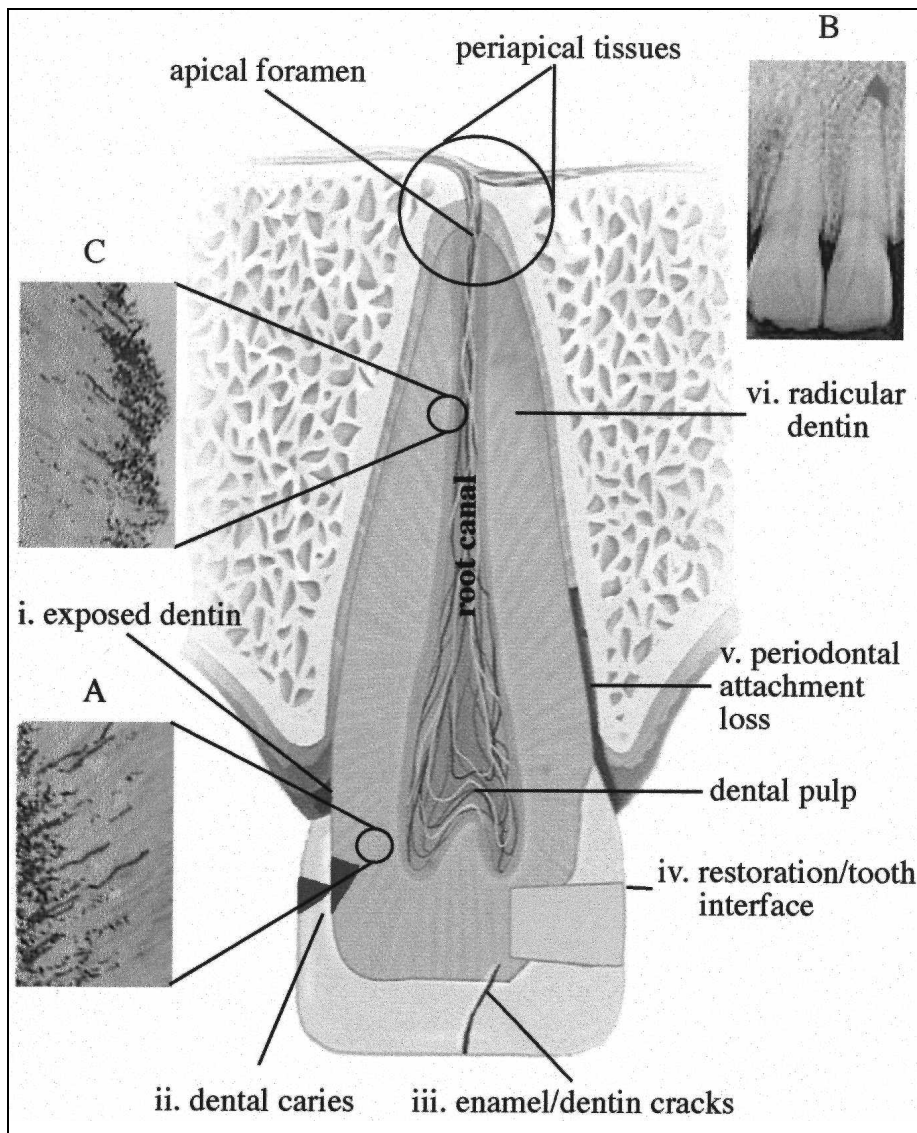


Figure 1. Common sites of bacterial invasion of dentin. Bacteria invading from the oral cavity (i, ii, iii, iv, v) extend toward the dental pulp space (A) and may result in inflammatory disease and infection of the pulp and periapical tissues. (B) Periapical radiograph demonstrating chronic periapical periodontitis of an upper left central incisor subsequent to infection of the root canal via an enamel-dentin crack. Bacteria invading radicular dentin (v) from an infected root canal invade outward toward the external root surface (C) and may be responsible for persistent root canal infection and inflammatory disease of the surrounding tissues. (Reprinted and modified with permission from Love, 1997.)

Invasion of dentinal tubules by bacteria from supra- or subgingival plaque occurs whenever dentin is exposed in the oral cavity. This can be through caries lesions, restorative or periodontal procedures, tooth wear, enamel or dentin cracks, or dental trauma (Tronstad and Langeland, 1971; Pashley, 1990; Peters *et al.*, 1995; Love, 1996a). Bacteria present within coronal dentinal tubules may be responsible for pulp and periapical disease (Brännström and Nyborg, 1971) (Fig. 1A), while those within radicular dentinal tubules may be responsible for continued root canal infection (Haapasalo and Ørstavik, 1987) (Fig. 1C).

Dental caries involving the crown of the tooth can affect people at any age from when the crown erupts into the mouth. By contrast, root-surface caries occurs only when

there has been loss of periodontal attachment and exposure of cementum or radicular dentin; hence it affects mainly adults. Unchecked, the advancing bacterial front of the carious process will result in infection of the dental pulp and root canal system, which will lead to periapical disease. However, bacteria that are associated with an infected root canal differ from those primarily associated with dental caries. Thus, although streptococci and *Actinomyces* are major components of dental plaque (Jenkinson and Lamont, 1997) and may initiate tubule and pulpal infection, obligately anaerobic bacteria are commonly present in large numbers in the infected root canal.

Streptococci are the primary bacterial colonizers of the oral cavity, and adhesion of streptococci to the acquired pellicle is an essential first step in colonization of the tooth (Gibbons, 1989; Kolenbrander and London, 1993; Jenkinson and Lamont, 1997). Streptococci express multiple surface protein adhesins (Hasty *et al.*, 1992) that allow cells to bind to a wide range of substrates found in the oral cavity, including other microbial cells, salivary components, host cells, or extracellular matrix or serum components (Jenkinson and Lamont, 1997). However, while there are considerable data on the mechanisms involved in the formation and development of dental plaque (Kolenbrander, 2000), relatively little is known about the mechanisms by which oral bacteria penetrate or invade dentin, and cause pulpitis, root canal infection, and periapical diseases. Advances in microbial sampling methods, and in growth and identification techniques, have provided much new information on the microbial components and complexes that are associated with endodontic and periodontal infections (Sundqvist, 1994; Socransky *et al.*, 1998). This article will review current knowledge of the microbiology of dentinal tubule infections. It will also describe how recent developments have advanced our understanding of the microbial complexity of root canal and pulpal infections, and of the mechanisms by which some species of oral bacteria are able to invade dentin.

(II) Microbiology of Infection of the Pulpo-Dentin Complex

(A) PULPO-DENTIN COMPLEX

Biologically and developmentally, pulp and dentin function as a complex and may be regarded as one tissue. Dentinal fluid movement, resulting in hydrodynamic activation of pulpal A-delta nerve fibers and causing dentin sensitivity (Brännström, 1986), is a common example of functional coupling of the tis-

sues. Both tissues are derived from the dental papilla, and development of the two tissues is closely related. The structure and composition of dentin matrix, and of the dentinal tubules, are key influences in the process of bacterial invasion of dentinal tubules.

The dental pulp is encased by dentin and occupies a space commonly designated the pulp chamber in coronal dentin and the root canal in radicular dentin. Dentin is porous, hard, mineralized connective tissue composed primarily of hydroxyapatite-coated collagen type I fibrils. Other collagen types (III, V, and VI) and non-collagenous proteins and proteoglycans are present as minor components. The matrix is formed by pulp odontoblast cells, which begin secreting collagen at the dentino-enamel junction and then retreat centripetally, trailing odontoblast processes around which the dentin matrix is elaborated and mineralized. This results in primary and secondary dentin having a tubular nature. Tertiary or reparative dentin, which is laid down as a consequence of noxious stimuli, does not have a regular tubular form. Since the circumference of the peripheral part of the crown or root is larger than the circumference of the final pulp chamber or root canal space, the odontoblasts are forced closer together as they continue to lay down intertubular dentin. This results in changes in the relative proportions of dentinal tubules within different areas of the dentin and a characteristic S-shape course of the dentinal tubules. The number of dentinal tubules *per* mm² varies from 15,000 at the dentino-enamel junction to 45,000 at the pulp (Garberoglio and Brännström, 1976). Deposition of intratubular (peritubular) dentin within the tubule results in narrowing of the tubule (Linde and Goldberg, 1993). Deposition is more advanced in superficial older dentin compared with dentin closer to the pulp, and this results in a tapered tubule with the largest dimensions at the pulp (approximately 2.5 μ m in diameter) and the smallest dimensions at the dentino-enamel or dentino-cemental junction (approximately 0.9 μ m in diameter) (Fig. 2). Thus, a tubule is normally larger in diameter than an average oral streptococcal cell (0.5-0.7 μ m).

Intratubular dentin is highly mineralized (approximately 95 vol% mineral phase) compared with the less-mineralized collagen matrix (about 30 vol% mineral phase) of intertubular dentin (Marshall, 1993), and becomes more mineralized with increasing age. This results in a decrease in size, and ultimately obliteration, of the dentinal tubules, with about 40% decrease in the overall numbers between the ages of 20 and 80 years (Tronstad, 1973; Carrigan *et al.*, 1984). The mean numbers of tubules at any given age within coronal, cervical, and mid-root dentin are similar (approximately 44,243, 42,360, and 39,010 mm⁻², respectively) (Carrigan *et al.*, 1984). However, significantly fewer dentinal tubules are found in apical dentin (approximately 8190 mm⁻²), suggesting that the formation of intratubular dentin occurs more rapidly in the apical region of the root.

(B) INTRATUBULAR CONTENT AND DIFFUSION PROPERTIES

The composition of dentinal tubule fluid in vital dentin is not fully known; however, it resembles serum with proteins

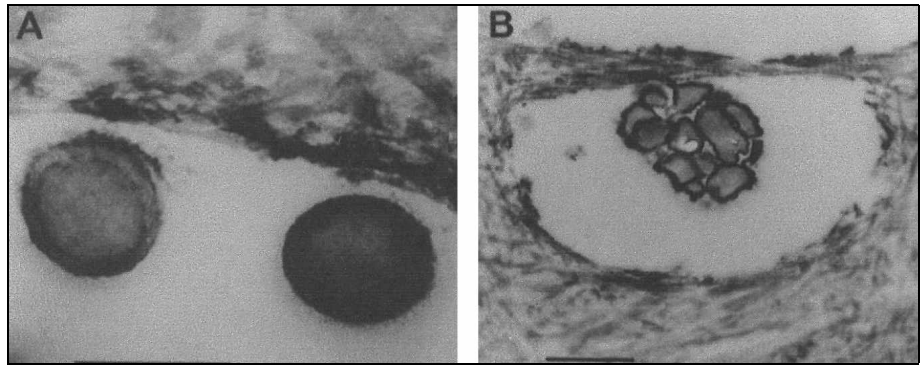


Figure 2. Transmission electron micrographs of sections of dentin colonized by *S. gordonii*. (A) Individual bacterial cells adhering to the wall of a dentinal tubule, with fibrillar surface material visible at the site of association between bacterial cells and tubule. Bar: 0.5 μ m. (B) A group of streptococcal cells in intimate contact with a tubule wall. Bar: 1.0 μ m. (Reproduced with permission from Love *et al.*, 1997.)

such as albumin and immunoglobulin G (IgG) being present (Knutsson *et al.*, 1994). In addition, other blood proteins, such as fibrinogen, may be found in dentinal tubules after cavity preparation (Knutsson *et al.*, 1994; Izumi *et al.*, 1998). Dentinal fluid within non-vital root dentin is fluid originating from alveolar bone and periodontal ligament, while dentinal fluid within non-vital coronal dentin is likely to be derived from saliva.

Dentinal tubules may contain odontoblast processes, nerve fibers, and unmineralized collagen fibrils. Dai *et al.* (1991) examined the contents of dentinal tubules of permanent human incisor, canine, premolar, and molar teeth from patients whose ages ranged from 18 to 54 yrs. They found that unmineralized collagen was a major component within dentinal tubules, occurring in 65% of all tubules in inner dentin (closest to the pulp). In 16% of these tubules, the collagen was aggregated into large bundles that occupied more than one-fifth of the lumen. In middle dentin, the corresponding figures were 42 and 7%, and for outer dentin, 12 and 0%. These patterns of collagen distribution were similar for all tooth families and were unrelated to age, suggesting that collagen is continually laid down within dentinal tubules throughout life.

Dentin is very porous because of the tubular structure. However, the degree of permeability varies between different areas of a tooth and the number of patent dentinal tubules present (Pashley, 1990). The pulpo-dentin complex is normally protected from the oral cavity by the overlying enamel or cementum. Once caries, trauma, or restorative or periodontal procedures breach the integrity of this barrier, the tubules provide diffusion channels from the surface to the pulp. Bacteria can then invade these dentinal tubules, and bacterial products can diffuse across dentin to elicit pulpal reactions (Vojinovic *et al.*, 1973; Bergenholtz, 1981). The pulp responds initially by mounting an inflammatory response that increases the outward flow of dentinal fluid (Maita *et al.*, 1991; Vongsavan and Matthews, 1994), thereby reducing diffusion of noxious stimuli through the dentinal tubules. Molecules present within dentinal tubules such as albumin, fibrinogen, and IgG have been shown to decrease fluid flow through dentin *in vitro* (Pashley *et al.*, 1982; Hahn and Overton, 1997). It is therefore likely that dentinal fluid components are involved in host defense, by both interacting directly with bacteria and products, and by reducing the permeability of dentin.

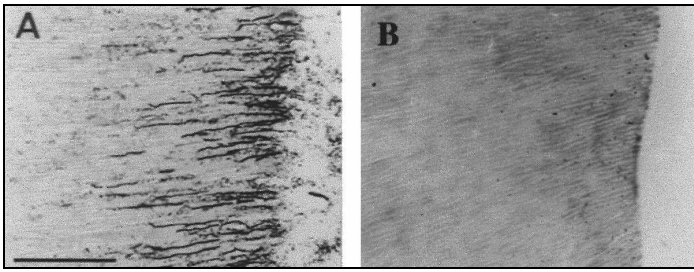


Figure 3. Transverse sections of human roots showing: (A) invasion of dentinal tubules by *S. gordonii* wild-type cells; and (B) no dentinal tubule invasion by *S. gordonii* in the presence of acid-soluble collagen type I. Bar: 50 μm . (Reproduced in modified form with permission from Love *et al.*, 1997.)

However, conditions that reduce the outward flow of dentinal fluid tend to increase the inward diffusion of exogenous substances. Pashley (1992) speculated that bacterial invasion of dentinal tubules would interfere more with outward fluid flow than with inward diffusion of noxious materials, due to the higher sensitivity of bulk fluid movement to changes in tubule radius, r (which varies with r^4), compared with diffusion (which varies with r^2). *In vitro* studies have demonstrated that fluid flow through dentin is indeed reduced by bacterial invasion of dentin (Michelich *et al.*, 1980; Love *et al.*, 1996). Reduced fluid flow might promote disease pathogenesis by allowing for an increased diffusion rate of destructive or toxic bacterial products toward the pulp. Continued stimulus results in the pulpo-dentin complex responding to the noxious challenge by activation of immunocompetent cells and inflammatory processes in the pulp and by decreasing the permeability of the dentin by the production of sclerotic or reparative dentin (for reviews, see Pashley, 1996; Jontell *et al.*, 1998). When unchecked, bacterial invasion of dentinal tubules overcomes the pulpo-dentin defenses, resulting in infection of the pulp and root canal system.

(III) Bacterial Invasion of Coronal Dentin

(A) ARIOUS DENTIN

The cariogenic microflora present on the surface of fissure, smooth-surface coronal, or root-surface caries consists mainly of streptococci, lactobacilli, and *Actinomyces* spp. Members of the mutans group streptococci, in particular *S. mutans* and *S. sobrinus*, are considered to be the primary etiological agents in the induction of coronal and of root caries (Bowden, 1990; van Houte, 1994; van Houte *et al.*, 1994). Samples of carious dentin from the outer surfaces of teeth contain *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp. and other Gram-positive rods (Loesche and Syed, 1973). Samples from the pulpal side of carious dentin lesions of extracted teeth contain larger numbers of Gram-positive anaerobic rods of *Eubacterium*, *Propionibacterium*, and *Bifidobacterium* species, with *Actinomyces* and *Lactobacillus* being the most prevalent facultative bacteria isolated (Edwardsson, 1974). In these studies, streptococci constituted only a minor group of the total isolates. Thus, different regions of carious dentin may contain quite different proportions of bacterial components in their microflora.

Greater numbers of bacteria are recovered from superficial infected dentin compared with deeper dentin (Hoshino, 1985). The application of strict anaerobic sampling and cultivation methods always gives higher recoveries of bacteria, implying that the environment of carious dentin promotes survival of obligately anaerobic bacteria. Thus, species of *Propionibacterium*, *Eubacterium*, and *Bifidobacterium* dominate the microflora of deep carious dentin, with *Actinomyces*, *Lactobacillus*, and some streptococci, but rarely *S. mutans*, being present (Table 1). Gram-negative obligate anaerobes, *e.g.*, *Fusobacterium*, are recovered in only very low numbers, if at all (Table 1). To identify and localize bacterial species within carious dentin, Ozaki *et al.* (1994) detected, by immunohistochemical techniques, specific bacteria within dentin samples from fissure, smooth-surface coronal, and root-surface caries. They found that mutans group streptococci were the predominant bacteria within dentin from fissure and smooth-surface coronal caries, with higher numbers in the shallow and middle layers of dentin compared with deep dentin. Other bacteria previously identified as being dominant members of the microflora of carious human dentin—such as *Lactobacillus* spp., *Eubacterium alactolyticum*, and *F. nucleatum* (Edwardsson, 1974; Hoshino, 1985)—were frequently detected, though their relative proportions were low (Table 1). Thus, the environment within superficial carious dentin favors growth of facultative anaerobes that are associated with the carious process, *e.g.*, mutans streptococci, while the microflora deep within the dentin is dominated by obligately anaerobic organisms.

In contrast to the microflora of fissure and smooth-surface carious dentin, *Actinomyces naeslundii (viscosus)* is the major species associated with dentin invasion in root-surface caries. *Actinomyces* species are found in shallow, middle, and deep dentin, with higher numbers of cells in deeper dentin. Mutans streptococci are frequently detected at all levels of carious root dentin, though they are mainly located in the shallow layer and do not make up a high proportion of the microflora. On the other hand, lactobacilli and Gram-negative organisms are found in low numbers, or not at all (Syed *et al.*, 1975; Hill *et al.*, 1977; Ozaki *et al.*, 1994) (Table 2). Thus, the composition of the microflora associated with carious dentin differs quite considerably between coronal and root caries.

(B) NON-CARIOUS DENTIN

In vivo studies show that bacteria are able to penetrate the tubules of non-cariou coronal dentin exposed to the oral environment. Invasion of tubules occurs readily and is evident within a week of exposure (Lundy and Stanley, 1969; Olgart *et al.*, 1974). With time, the numbers of tubules infected and the depth of infection increase (Lundy and Stanley, 1969). The pattern of invasion is characterized by variable numbers of tubules penetrated and variable depths of penetration between different areas of dentin (Fig. 3A) (Tronstad and Langeland, 1971; Olgart *et al.*, 1974). Inflammatory changes within the pulp are commonly observed and can be seen within a week of exposure (Olgart *et al.*, 1974). Other studies have demonstrated that microleakage of oral bacteria around restorations allows for bacterial invasion of exposed dentinal tubules at the base of the cavity (Brännström and Nyborg, 1971; Vojinovic *et al.*, 1973), resulting in pulpal inflammation (Vojinovic *et al.*, 1973) or periapical disease (Ray and Trope, 1996). Likewise, microleakage through enamel cracks and fractures as a result of trauma may lead to bacterial invasion of

TABLE 1
Bacterial Species Identified in Carious Coronal Dentin

Bacterial Genus or Species	Isolation Frequency in Carious Dentin		Bacterial Genus or Species	Isolation Frequency in Carious Dentin	
	Superficial	Deep		Superficial	Deep
<i>Streptococcus</i> <i>S. mutans</i> <i>S. sobrinus</i> <i>S. intermedius</i> <i>S. morbillorum</i> <i>S. sanguinis</i>	High	Low-moderate	<i>Propionibacterium</i> <i>P. acnes</i> <i>P. avidum</i> <i>P. lymphophilum</i> <i>P. propionicum</i>	Moderate-high Low	High Moderate
<i>Peptostreptococcus</i> <i>P. anaerobius</i> <i>P. parvulus</i> <i>P. micros</i>	Low	<i>Lactobacillus</i> Low	High <i>L. casei</i> <i>L. plantarum</i> <i>L. minutus</i>		High
<i>Actinomyces</i> <i>A. israelii</i> <i>A. naeslundii</i> <i>A. odontolyticus</i>	High	<i>Fusobacterium nucleatum</i> Moderate	Low <i>Bifidobacterium</i> spp. <i>Peptococcus</i> spp.	Low High Low	 High Low
<i>Eubacterium</i> <i>E. alactolyticum</i> AQ <i>E. aerofaciens</i> <i>E. saburreum</i>	High	<i>Clostridium</i> spp. High	Low <i>Porphyromonas</i> spp. <i>Prevotella</i> spp.	Low Low Low	 Low Low
<i>Veillonella</i> spp.	Moderate	Low			

Modified from Edwardsson, 1987; Ozaki *et al.*, 1994.

TABLE 2
Bacterial Species Identified in Carious Root Dentin

Bacterial Species	Isolation Frequency	Bacterial Species	Isolation Frequency
<i>Streptococcus</i> <i>S. sanguinis</i> <i>S. mitis</i> <i>S. mutans</i> <i>S. sobrinus</i>	Low-high	<i>Propionibacterium</i> spp. e.g., <i>P. acnes</i>	Low-moderate
	<i>L. plantarum</i>	<i>Lactobacillus</i> <i>L. casei</i>	Low
<i>Actinomyces</i> <i>A. naeslundii</i> <i>A. odontolyticus</i> <i>A. viscosus</i>	High	<i>Peptostreptococcus micros</i> <i>F. nucleatum</i> <i>P. endodontalis</i>	Low Low
<i>Eubacterium</i> spp. e.g., <i>E. alactolyticum</i>	Low-moderate	<i>Veillonella</i> spp.	Low

Modified from Edwardsson, 1987; Ozaki *et al.*, 1994.

TABLE 3**Bacterial Species Commonly Found in Asymptomatic Infected Root Canals**

<u>Gram-positive Cocci</u>	<u>Gram-positive Rods</u>
<i>Streptococcus anginosus</i>	<i>Actinomyces israeli</i>
<i>S. sanguinis</i>	<i>A. naeslundii</i>
<i>S. mitis</i>	
<i>S. mutans</i>	<i>Eubacterium alactolyticum</i>
	<i>E. lentum</i>
<i>Enterococcus faecalis</i>	<i>E. nodatum</i>
	<i>E. timidum</i>
<i>Peptostreptococcus micros</i>	
<i>P. anaerobius</i>	<i>Propionibacterium propionicum</i>
	<i>P. granulosum</i>
	<i>Lactobacillus</i>
<u>Gram-negative Cocci</u>	<u>Gram-negative Rods</u>
<i>Capnocytophaga ochracea</i>	<i>Fusobacterium nucleatum</i>
<i>C. sputigena</i>	
	<i>Prevotella intermedia</i>
	<i>P. melaninogenica</i>
<i>Veillonella parvula</i>	<i>P. denticola</i>
	<i>P. buccae</i>
	<i>P. buccalis</i>
<i>Campylobacter rectus</i>	<i>P. oralis</i>
<i>C. curvus</i>	
	<i>Porphyromonas gingivalis</i>
	<i>P. endodontalis</i>
	<i>Bacteroides gracilis</i>

Adapted from Sundqvist, 1992a,b, 1994; Le Goff *et al.*, 1997.

the pulpo-dentin complex and act as a cause of pulpal disease (Love, 1996a). Hence, sealing of dentin from exogenous substances and bacteria in the oral cavity, in both vital and non-vital teeth, is a critical step in tooth restoration.

The composition of the microflora invading exposed non-carious dentin has not been fully elucidated but is dominated by Gram-positive cells (Lundy and Stanley, 1969; Brännström and Nyborg, 1971; Tronstad and Langeland, 1971; Vojinovic *et al.*, 1973; Olgart *et al.*, 1974) and probably resembles the composition of the biofilm infiltrating the tooth-restoration interface (Edwardsson, 1987). This biofilm resembles mature plaque and is composed mainly of streptococci and *Actinomyces* spp. Anaerobic Gram-positive cocci, *e.g.*, *Peptostreptococcus micros*, and Gram-negative organisms tend to be present in only low numbers (Mejäre *et al.*, 1979, 1987).

(IV) Microflora of the Infected Root Canal

Bacteria may enter the root canal system directly *via* caries lesions or *via* pulp exposure following trauma. However, many infections of the pulp occur as a result of supra- or sub-gingival bacteria penetrating exposed dentin, enamel-dentin cracks, and around restorations (Pashley, 1990; Peters *et al.*, 1995; Love, 1996a) and then invading dentinal tubules. Almost all bacteria recovered from the root canal systems of teeth with intact crowns belong to the oral microflora (Wittgow and Sabiston, 1975; Sundqvist, 1976; Le Goff *et al.*, 1997). The con-

cept that bacteria can gain access to the pulp system *via* the blood stream has not been proven. In fact, Delivanis and Fan (1984) were unable to demonstrate the presence of bacteria in unfilled cat root canals after repeated intravenous injections of *S. sanguis* (*sanguinis*). More than 300 bacterial species are recognized as components of the oral microflora (Moore and Moore, 1994). However, only relatively few species appear to be able to invade the root canal space and infect the root canal (Kantz and Henry, 1974; Sundqvist, 1976; Dahlén and Bergenholtz, 1980). This suggests that many species of oral bacteria do not have the properties necessary to invade tubules and survive within the intratubular environment.

In studies where strict avoidance of contamination is attempted, sampling has been done of teeth with intact pulp chamber walls (Sundqvist, 1976). Consequently, the bacteria that are detected in the root canal must have gained entry by invading dentinal tubules. Sundqvist (1976) studied the microflora of human teeth that had become non-vital as a result of trauma, but which otherwise were intact and caries-free. Utilizing strictly anaerobic sampling techniques, he demonstrated that bacteria could not be isolated from teeth with normal periapical tissue, while bacteria were regularly isolated from teeth from patients who had apical periodontitis. Likewise, Möller *et al.* (1981) showed that only devitalized and infected pulps of monkey teeth showed signs of apical periodontitis, whereas devitalized and uninfected pulps did not develop periapical bone destruction. The pioneering studies by Sundqvist (1976) and later by Möller *et al.* (1981) demonstrated that, in addition to streptococci, lactobacilli, and *Actinomyces*, obligately anaerobic species of *Fusobacterium*, *Peptostreptococcus*, *Eubacterium*, *Propionibacterium*, *Veillonella*, *Wolinella*, *Prevotella*, and *Porphyromonas* dominated the root canal microflora (Table 3). Other micro-organisms such as yeasts, *e.g.*, *Candida* and *Saccharomyces* (Lana *et al.*, 2001), and spirochetes, *e.g.*, *Treponema* (Jung *et al.*, 2000; Rôças *et al.*, 2001), have been occasionally recovered from an infected root canal. Most of the oxygen-sensitive members of the root canal microflora are not readily cultivable without the strict application of anaerobic methods (Carlsson *et al.*, 1977), and this may explain why, in earlier studies, many teeth with apical periodontitis did not appear to harbor bacteria in the root canal.

Obligately anaerobic bacteria dominate the microflora of established asymptomatic infected root canals, with streptococci making up a significant proportion of the facultative species. Commonly, between 2 and 8 bacterial species are recovered from infected root canals, with *F. nucleatum*, *P. intermedia*, and streptococci being often present (Sundqvist, 1994; Le Goff *et al.*, 1997) (Table 3). A series of studies on the dynamics of experimental root canal infections of monkey teeth has shown that facultative anaerobic bacteria, mainly streptococci, are the first colonizers of the root canal, but that by 6 months, obligate anaerobes dominate the microflora. When combinations of bacterial strains, isolated originally from an endogenously infected root canal, were re-inoculated into further canals with devitalized tissue, the dominance of anaerobic bacteria was again established. Furthermore, the original proportions of the bacterial strains were re-established, despite equal numbers of the different strains being inoculated into the canals (Fabricius *et al.*, 1982a,b). These observations support the notion that associations between specific bacteria enable the root canal microflora to grow and survive in a highly specialized and selective environment (Sundqvist, 1992a).

Mixed root canal infections result in larger periapical lesions than do mono-infections (Fabricius *et al.*, 1982a,b). However, while the components of the root canal microflora are well-established, it is interesting that no single bacterial species has been indicted as the major pulp and periapical pathogen in chronic asymptomatic conditions. *P. gingivalis*, which is strongly implicated in destructive adult periodontal disease (Socransky and Haffajee, 1992; Lamont and Jenkinson, 1998), is recovered in low numbers from asymptomatic chronic root canal infections (Sundqvist, 1994; Le Goff *et al.*, 1997). However, numbers of *Porphyromonas* and *Prevotella* species increase dramatically when there are signs and symptoms of acute periapical infection (Haapasalo, 1989; Sundqvist *et al.*, 1989; Hashioka *et al.*, 1992). The dominance of Gram-negative species in the latter stages of root canal infection supports the evidence that a highly selective environment continues to develop within the root canal system. Moreover, mechanisms may exist that allow these Gram-negative obligate anaerobes, *e.g.*, *Porphyromonas* and *Prevotella* species, to penetrate dentin, even though the bacteria are not routinely isolated from the tubule microflora.

The microflora of carious and cavitated dentin of teeth with pulpitis is similar to that previously reported for intact carious dentin (Hahn *et al.*, 1990) (Table 1). Gram-positive organisms predominate, especially *Lactobacillus* spp. and streptococci. Gram-negative bacteria, *e.g.*, *P. intermedia*, are found in lower numbers in superficial to deep dentin, but are more prevalent within dentin at the pulpal wall. Investigating the degree of cellular infiltrate and degenerative changes in the pulps of teeth with cavitated carious dentin, Massey *et al.* (1993) reported no association between the microbial load within the dentin and histopathology of the pulp. However, there was a positive correlation between the presence of *P. intermedia* and *P. melaninogenica* and extensive inflammation of the pulp.

(V) Bacterial Invasion of Radicular Dentin from the Root Canal

Once bacteria gain access to the root canal system, they invade root canal dentinal tubules (Fig. 1C) and may be responsible for persistent root canal infection (Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990). Shovelton (1964) examined histologically 97 extracted, clinically non-vital teeth and found that 61 of the teeth showed bacterial penetration of the radicular dentinal tubules. The numbers of tubules containing bacteria were highly variable from tooth to tooth and among sections of an individual tooth. The depth of penetration by bacteria into the tubules was also found to be variable. It was noted that the presence of bacteria within the tubules was related to the clinical history of the tooth, such that chronic infections had more bacterial invasion and that tubule invasion did not occur immediately after the bacteria appeared in the root canal. These observations were similar to those reported in later histological studies on the invasion of non-carious coronal dentin (Lundy and Stanley, 1969; Brännström and Nyborg, 1971; Tronstad and Langeland, 1971; Vojinovic *et al.*, 1973; Olgart *et al.*, 1974).

The microflora within radicular dentinal tubules of teeth with infected root canals (Ando and Hoshino, 1990) resembles that of deep layers of carious coronal dentin (Edwardsson, 1974; Hoshino, 1985) (Table 1). Lactobacilli, streptococci, and *Propionibacterium* spp. are predominant, with other bacteria

such as Gram-positive anaerobic cocci, *Eubacterium* spp., and *Veillonella* spp. being present in low numbers. Obligately anaerobic Gram-negative bacteria were recovered in very low numbers or not at all (Edwardsson, 1974; Hoshino, 1985; Ando and Hoshino, 1990), but are known to be present in infected root canals, as previously discussed. The inability to detect fastidious anaerobes within invaded coronal or radicular dentin may have been due simply to difficulties in cultivating these bacteria. By utilizing specific antisera, Ozaki *et al.* (1994) demonstrated that *P. endodontalis* was present, albeit in low numbers, within dentinal tubules of carious dentin. Recently, Peters *et al.* (2001) demonstrated that the flora recovered from mid-root radicular dentin of teeth with apical periodontitis of endodontic origin was similar to that reported in previous studies (Ando and Hoshino, 1990), while Gram-negative bacteria including *F. nucleatum*, *P. gingivalis*, and *P. intermedia* were commonly recovered. Clearly, Gram-negative obligate anaerobic bacteria are more frequently found, and in higher cell numbers, in infected root canals than in carious and non-carious infected dentin. Undoubtedly, the application of novel molecular techniques that detect bacteria in samples without the necessity for laboratory cultivation (Dymock *et al.*, 1996), or the presence of bacteria *in situ*, will assist greatly in future analyses of infected dentin, root canals, and pulpal tissues.

(VI) Bacterial Invasion of Radicular Dentin from a Periodontal Pocket

Bacterial invasion of radicular dentin of periodontally diseased teeth has been demonstrated by light microscopy (Kopczyk and Conroy, 1968; Langeland *et al.*, 1974; Adriaens *et al.*, 1987b) and by microbiological studies (Adriaens *et al.*, 1987a; Giuliana *et al.*, 1997). It has been suggested that the dentinal tubule microflora associated with a periodontal pocket could act as a reservoir for re-colonization of the pocket after debridement (Adriaens *et al.*, 1987a; Giuliana *et al.*, 1997). The majority of species recovered from radicular dentin are Gram-positive bacteria (*P. micros*, *S. intermedius*, *A. naeslundii*), with lower numbers of Gram-negative organisms (*P. gingivalis*, *P. intermedia*, *Bacteroides forsythus*, *F. nucleatum*, *V. parvula*) (Giuliana *et al.*, 1997).

While it is clear that bacteria are able to invade radicular dentin from the periodontal pocket, a contentious issue is whether bacteria invade healthy cementum prior to dentin penetration, or if bacteria gain access to dentin only *via* breaches in the cementum layer. Several studies have described invasion of the cementum of periodontally diseased teeth (Hartzell, 1911; Daly *et al.*, 1982; Adriaens *et al.*, 1987a,b; Giuliana *et al.*, 1997). However, it was not evident from any of these studies if the invaded cementum was intact, healthy, or diseased. Exposed cementum is a thin, often discontinuous layer (Moskow, 1969), and commonly shows surface defects, *e.g.*, at sites where Sharpey's fibers attach to the cementum matrix (Adriaens *et al.*, 1987b). Exposure of cementum to crevicular fluid, bacterial enzymes, or acidic metabolites may induce physicochemical and structural alterations, such as localized resorptive lacunae or demineralization (Daly *et al.*, 1982; Eide *et al.*, 1984; Adriaens *et al.*, 1987b). It seems likely, therefore, that bacterial invasion of exposed cementum associated with periodontal disease occurs after the cementum has been altered by physiological, bacterial, or environmental factors.

TABLE 4**Bacteria Associated with *in vivo* Dentin Caries and Root Canal Infection that can Invade Root Dentinal Tubules *in vitro***

Bacterium	Reference
<i>Streptococcus sanguinis</i>	Akpata and Blechman, 1982 Ørstavik and Haapasalo, 1990 Perez <i>et al.</i> , 1993
<i>Streptococcus gordonii</i>	Love, 1996b Love <i>et al.</i> , 1996 Love <i>et al.</i> , 1997
<i>Enterococcus faecalis</i>	Akpata and Blechman, 1982 Haapasalo and Ørstavik, 1987 Ørstavik and Haapasalo, 1990
<i>Streptococcus sobrinus</i>	Nagaoka <i>et al.</i> , 1995
<i>Lactobacillus casei</i>	Nagaoka <i>et al.</i> , 1995
<i>Actinomyces viscosus (naeslundii)</i>	Nagaoka <i>et al.</i> , 1995
<i>Streptococcus mutans</i>	Love <i>et al.</i> , 1997

(VII) Bacterial Invasion *in vitro*

In vitro studies have examined penetration of coronal or root dentin by a limited number of oral bacteria that are associated with carious or non-carious dentin. Cells of *S. mutans*, *S. sanguinis*, and *A. naeslundii* have all been shown to penetrate dentin discs *in vitro* (Michelich *et al.*, 1980; Meryon *et al.*, 1986; Meryon and Brook, 1990). Invasion of root dentinal tubules by pure cultures of streptococci or enterococci associated with root canal infections *in vivo*, or with dentinal caries, has been demonstrated histologically (see Fig. 3A) (Table 4). In contrast, invasion of dentin by mono-cultures of Gram-negative anaerobic bacteria is less clear, but invasion has not been generally recognized. Neither *Bacteroides melaninogenicus* ss. *melaninogenicus* nor *P. intermedia* (Akpata and Blechman, 1982; Perez *et al.*, 1993) invaded root dentin after 21-28 days' incubation. On the other hand, limited invasion by *P. intermedia* has been reported (Berkiten *et al.*, 2000), while *P. endodontalis* and *P. gingivalis* both showed low-level penetration of dentinal tubules of bovine roots that had the cementum removed (Siqueira *et al.*, 1996).

The ability of mixed cultures of bacteria, associated with coronal or root caries, to invade dentin was investigated by Nagaoka *et al.* (1995). Analysis of their data suggested that invasion of *L. casei* was enhanced when co-cultured with *S. sobrinus* or *A. naeslundii*. More recently, it has been shown that dentinal tubule invasion by *P. gingivalis* was promoted when co-cultivated with *S. gordonii* (Love *et al.*, 2000). These experiments demonstrate that bacteria may compete for invasion of dentinal tubules, and also that they may co-operate in invasion. Both these interactions may be significant in determining the outcome of tubule infections.

(VIII) Factors Influencing Tubule Invasion by Bacteria**(A) DENTIN STRUCTURE**

Whenever dentin is cut or abraded, a smear layer of debris forms on the instrumented surface and packs into the superficial portion of the dentinal tubule. *In vitro* experiments suggest that the presence of a dentinal smear layer prevents the pene-

tration of coronal or root dentinal tubules by streptococci (Michelich *et al.*, 1980; Love *et al.*, 1996), and this is confirmed by *in vivo* studies. Bacterial invasion of dentinal tubules occurs more readily when the smear layer has been removed from the dentin, compared with smeared dentin where the degree of tubule invasion is low (Vojinovic *et al.*, 1973; Olgart *et al.*, 1974). Additionally, the degree of pulp inflammation appears less pronounced under smeared dentin.

Depth of bacterial invasion may depend, at least in part, upon tubule diameter, since this determines the rate of solute diffusion (Pashley, 1992). Sclerotic or obliterated tubules will physically impede bacterial invasion and can result in regional differences in bacterial invasion of dentin. Invasion of coronal and mid-root dentin occurs readily by *S. gordonii*, while the extent and depth of invasion are significantly less in apical dentin (Love, 1996b). This is because of the lower number of patent tubules in this region due to dentinal sclerosis, which is always more advanced in the apical region compared with coronal and mid-root dentin at any age.

Intact cementum is crucial to limitation of the bacterial invasion of radicular dentinal tubules from the pulpal surface. Penetration is enhanced when the overlying cementum is resorbed (Valderhaug, 1974; Haapasalo and Ørstavik, 1987; Love, 1996b), a common occurrence in the presence of inflammatory periapical disease and after traumatic injuries that damage the periodontal ligament.

Limiting nutritional supply may influence the depth of bacterial penetration. This is partly dependent upon the patency of the tubule, since diffusion of substances into tubules from the oral cavity or pulpal fluid is proportional to tubule diameter (discussed above). This may account for the higher numbers of cariogenic bacteria present within superficial dentin (Edwardsson, 1987), where the presence of fermentable carbohydrates and oxygen from the oral cavity is likely to be higher than in deeper dentin. Also, the anaerobic environment and the possible presence of tissue components, *e.g.*, hemin, within dentin close to the pulp is likely to favor growth and survival of organisms such as *P. intermedia* and *P. gingivalis* (Hahn *et al.*, 1990).

(B) BACTERIAL ADHESION

The pivotal nature of streptococcal interactions with deposited salivary proteins and glycoproteins on oral surfaces and other organisms is well-recognized in the development of the complex dental plaque biofilms (Gibbons, 1984; Malamud, 1985; Banas *et al.*, 1990; Terpenning *et al.*, 1993; Kolenbrander, 2000). A great many streptococcal protein adhesins have been identified that can interact with salivary molecules. These include the antigen I/II family polypeptides (Jenkinson and Demuth, 1997), amylase-binding proteins (Scannapieco, 1994), surface lectins (Murray *et al.*, 1986; Takahashi *et al.*, 1997), fimbrial adhesins (Oligino and Fives-Taylor, 1993; Wu and Fives-Taylor, 1999), EP-GP binding protein (Schenkels *et al.*, 1993), and glucan-binding proteins GBP₇₄ (Banas *et al.*, 1990) and GBP₅₉ (Smith *et al.*, 1994). The possession of multiple salivary adhesins favors colonization by a range of mechanisms. Interbacterial co-aggregation is also an important aspect in plaque development (Kolenbrander and London, 1993). Streptococci co-adhere with other early colonizers, such as *Actinomyces* spp., and are also bound by later colonizers such as *P. gingivalis* and *B. forsythus* (Lamont *et al.*, 1992; Yao *et al.*, 1996). Later colonizers are often strict anaerobes and increase in

plaque when a more anaerobic environment develops, which may be due, in part, to the actions of earlier colonizers. Despite our extensive knowledge about adhesive interactions between bacteria and substrates in the oral cavity, the influence of bacterial adhesion and inter-bacterial binding in tubule invasion is relatively poorly understood.

Collagen type I, a major organic component of dentin, is recognized by oral streptococci, and when absorbed onto hydroxyapatite surfaces, it serves as an adhesion substrate (Liu and Gibbons, 1990; Liu *et al.*, 1990). Strains of *S. mutans* are able to bind to unmineralized collagen and to particles of root dentin (Switalski *et al.*, 1993). The ability of oral streptococci to bind to collagen may facilitate bacterial adhesion to exposed dentin or cementum, and subsequently tissue penetration. The antigen I/II polypeptides, expressed on the surfaces of most indigenous species of oral streptococci (Jenkinson and Demuth, 1997), play a major role in mediating adhesion of streptococci to collagen (Love *et al.*, 1997). Strains of *P. gingivalis* also readily bind to collagen-coated hydroxyapatite, and to bovine bone collagen (Naito and Gibbons, 1988; Naito *et al.*, 1993). This binding is due, at least in part, to the adhesion fimbriae that bind strongly to collagen *in vitro* (Naito *et al.*, 1993). Fimbriae are involved in other adhesive interactions important in host colonization by *P. gingivalis*, such as binding to salivary receptors, epithelial cells, fibronectin, and other oral bacteria (Isogai *et al.*, 1988; Goulbourne and Ellen, 1991; Li *et al.*, 1991; Lamont and Jenkinson, 2000), and in the invasion of epithelial cells (Lamont *et al.*, 1995; Weinberg *et al.*, 1997).

Recent data have provided strong evidence for bacterial adhesion specificity as playing a major role in determining the invasion of dentinal tubules. Experiments utilizing isogenic mutants of *S. gordonii* or *S. mutans* deficient in the expression of antigen I/II polypeptide surface adhesins clearly demonstrate that these polypeptides not only mediate streptococcal binding to collagen, but also are necessary for bacterial invasion of dentin (Love *et al.*, 1997). It seems that recognition of type I collagen may facilitate bacterial adhesion to dentin (Fig. 2) as well as a morphological growth response manifested by long-chaining of streptococcal cells (Love *et al.*, 1997). In support of this suggestion, acid-soluble type I collagen fragments completely inhibit dentinal tubule penetration by streptococci *in vitro* (Fig. 3B). These and subsequent experiments with mixed cultures of oral bacteria have led to the following model (Fig. 4) for dentinal tubule invasion by streptococci and *P. gingivalis*. It is envisaged that antigen I/II family polypeptides produced by *S. gordonii*, *S. mutans*, and other oral streptococci mediate primary binding of bacteria to intratubular collagen type I. Streptococcal growth and

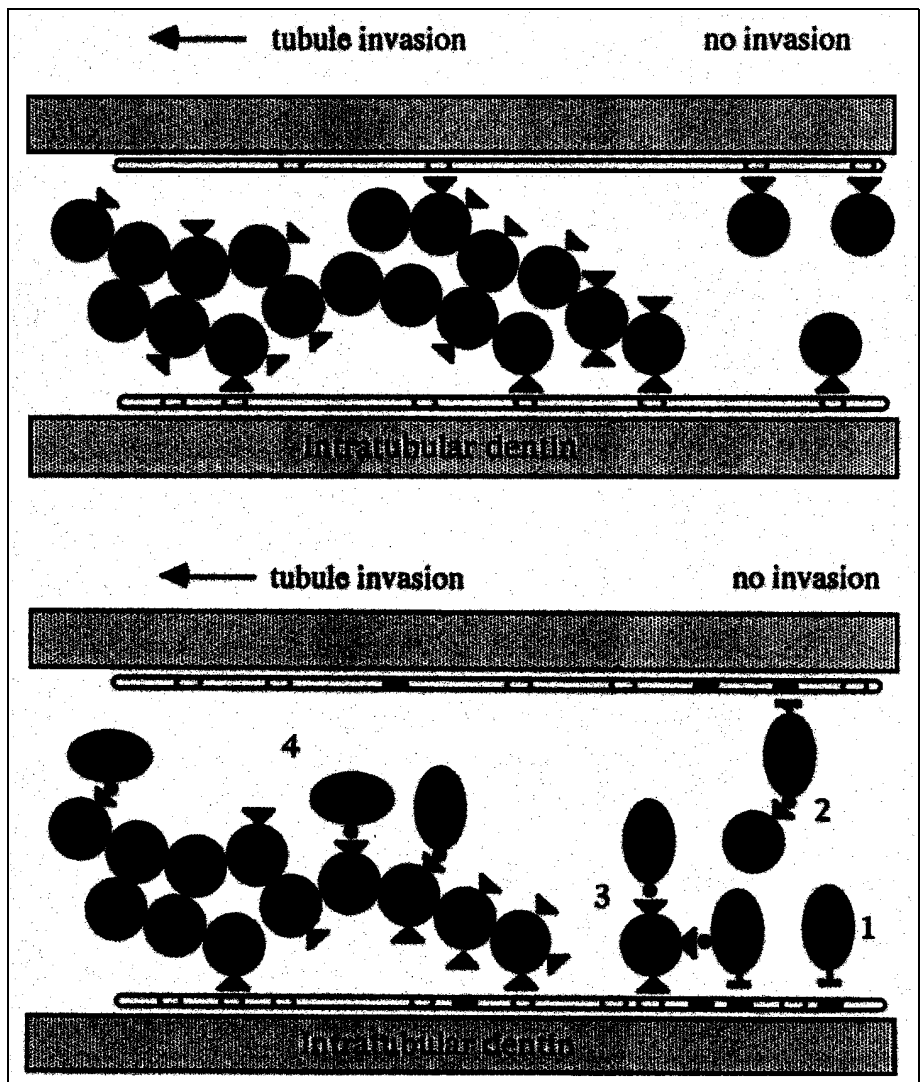


Figure 4. Streptococcal invasion of dentinal tubules (upper diagram) and co-invasion with *P. gingivalis* (lower diagram). Streptococcal cells (●) adhere to unmineralized collagen type I (◻) via antigen I/II polypeptide adhesin (▲). Growth of streptococci in the presence of collagen peptides leads to up-regulation of antigen I/II production (▲), long-chaining of cells, and colonization along the length of the tubule. In the lower diagram, *P. gingivalis* cells (●) and *S. gordonii* cells both adhere to collagen (1), but *P. gingivalis* is unable to penetrate the tubules further in monoculture. The presence of *S. gordonii* (2) provides an additional binding substrate for *P. gingivalis* and promotes intratubular colonization by *P. gingivalis*. Up-regulation of streptococcal antigen I/II adhesin production (3) provides additional binding sites for *P. gingivalis*. These bacteria remain in association with the streptococci (4), and the dentinal tubules become invaded by a mixed bacterial population.

metabolism promotes localized demineralization together with release of collagen peptides. The presence of these peptides leads to up-regulation of antigen I/II polypeptide production (Love *et al.*, 1997), enhanced adhesion, and facilitates community growth within and along the dentinal tubules (Figs. 2, 4). While *P. gingivalis* cells are able to bind collagen, this is not sufficient in itself to promote tubule invasion by these organisms in mono-culture. However, when *P. gingivalis* cells are co-cultivated with *S. gordonii* cells, invasion by the porphyromonads is promoted. This appears to depend upon the specific adherent interaction between *S. gordonii* and *P. gingivalis* cells, mediated by the streptococcal antigen I/II

polypeptides (Love *et al.*, 2000). Invasion is not dependent upon production of major adhesion fimbriae by *P. gingivalis*, that bind collagen, since isogenic *P. gingivalis* mutants defective in major fimbriae are still able to co-invade with *S. gordonii* (Love *et al.*, 2000). On the other hand, the antigen I/II polypeptide SpaP of *S. mutans* binds only weakly to *P. gingivalis* cells, and *S. mutans* cells do not allow the invasion of dentinal tubules by *P. gingivalis* (Love *et al.*, 2000).

It is likely that other bacterial interactions between host proteins and other bacteria may influence tubule invasion. Recently, it has been demonstrated that dentinal tubule invasion and adhesion to collagen by *S. mutans* or *S. gordonii* were inhibited by human serum, suggesting a protective mechanism of serum (Love, 2001). In contrast, cells of *E. faecalis*, a species commonly recovered from the root canals of failed endodontic cases, maintained their ability to invade dentin and adhere to collagen in the presence of serum (Love, 2001). It was suggested that, following root canal therapy, this ability may allow residual *E. faecalis* cells in radicular dentin to re-colonize the obturated root canal and participate in chronic failure of endodontically treated teeth (Love, 2001).

Analysis of these data demonstrates that specific adherent interactions between oral bacteria may facilitate tubule invasion. The observations should stimulate more detailed investigations of other bacterial interactions and their role in determining the composition of the dentinal and root canal microflora and the outcome of endodontic infections.

(IX) Summary and Future Prospects

Bacterial invasion of dentinal tubules and the clinical consequences thereof have been recognized for over a century. However, while many components of the infected dentinal tubule microflora have been identified, it seems likely that there are etiological agents of endodontic infections that have not yet been recognized. Molecular techniques of identification and quantification will be powerful tools in future studies of endodontic infections. Bacterial invasion of dentin occurs rapidly once the dentin is exposed to the oral environment, and in the early stages of infection, Gram-positive plaque bacteria dominate the microflora. The identification of adhesins that mediate these initial interactions of bacteria with dentin is important for the design of adhesion-blocking compounds. For example, agents that block antigen I/II polypeptide recognition of collagen, or that block the co-adhesion-mediating properties of antigen I/II protein, could be effective in controlling or preventing the initial invasion of dentin *via* the dentinal tubules. With time, fastidious obligately anaerobic bacteria become established as principal components of the microflora and can be found within the deep dentin layers. Unchecked bacterial invasion leads to inflammatory pulp disease, root canal infection, and periapical disease. It is important, therefore, that the mechanisms of invasion and interbacterial adhesion at all stages of the process be understood if novel control strategies are to be developed. These might include compounds that are added to dentifrices or mouthwashes, or that could be incorporated into dental materials, to inhibit the bacterial invasion of dentinal tubules. With longer retention of dentition in populations in many regions of the world, and increased incidence of root exposure, it is likely that infections of the pulp and periapical disease will have wider clinical implications in the very near future.

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REFERENCES

- Adriaens PA, De Boever JA, Loesche WJ (1987a). Bacterial invasion in root cementum and radicular dentin of periodontally diseased teeth in humans. *J Periodontol* 59:222-230.
- Adriaens PA, Edwards CA, De Boever JA, Loesche WJ (1987b). Ultrastructural observations on bacterial invasion in cementum and radicular dentin of periodontally diseased human teeth. *J Periodontol* 59:493-503.
- Akpata ES, Blechman H (1982). Bacterial invasion of pulpal dentin wall *in vitro*. *J Dent Res* 61:435-438.
- Ando N, Hoshino E (1990). Predominant obligate anaerobes invading the deep layers of root canal dentine. *Int Endodont J* 23:20-27.
- Banas JA, Russell RRB, Ferretti JJ (1990). Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans* Ingbritt. *Infect Immun* 58:667-673.
- Bergenholtz G (1981). Inflammatory responses of the dental pulp to bacterial irritation. *J Endodont* 7:100-104.
- Berkiten M, Okar I, Berkiten R (2000). *In vitro* study of the penetration of *Streptococcus sanguis* and *Prevotella intermedia* strains into human dentinal tubules. *J Endodont* 26:236-239.
- Bowden GHW (1990). Microbiology of root surface caries in humans. *J Dent Res* 69:1205-1210.
- Brännström M (1986). The hydrodynamic theory of dentinal pain: sensation in preparations, caries, and the dentinal crack syndrome. *J Endodont* 12:453-457.
- Brännström M, Nyborg H (1971). The presence of bacteria in cavities filled with silicate cement and composite resin materials. *S* 64:149-155.
- Carlsson J, Frolander F, Sundqvist G (1977). Oxygen tolerance of anaerobic bacteria isolated from necrotic dental pulps. *Acta Odontol Scand* 35:139-145.
- Carrigan P, Morse JDR, Furst ML, Sinai IH (1984). A scanning electron microscope evaluation of human dentinal tubules according to age and location. *J Endodont* 10:359-363.
- Cowan M, Taylor MKG, Doyle RJ (1986). Kinetic analysis of *Streptococcus sanguis* adhesion to artificial pellicle. *J Dent Res* 65:1278-1283.
- Dahlén G, Bergenholtz G (1980). Endodontic activity in teeth with necrotic pulps. *J Dent Res* 59:1033-1040.
- Dai X-F, Ten Cate AR, Limeback H (1991). The extent and distribution of intratubular collagen fibrils in human dentine. *Arch Oral Biol* 36:775-778.
- Daly C, Seymour G, Kieser J, Corbet E (1982). Histological assessment of periodontally involved cementum. *J Clin Periodontol* 9:266-274.
- Dawes C, MacPherson LMD (1993). The distribution of saliva and sucrose around the mouth during the use of chewing gum and the implications for the site specificity of caries and calculus deposition. *J Dent Res* 72:852-857.
- Delivanis PD, Fan VSC (1984). The localisation of blood-borne bacteria in instrumented unfilled and overinstrumented canals. *J Endodont* 19:521-524.
- Duncan MJ, Nakao S, Skobe Z, Xie H (1993). Interactions of *Porphyromonas gingivalis* with epithelial cells. *Infect Immun* 61:2260-2265.
- Dymock D, Weightman AJ, Scully C, Wade WG (1996). Molecular analysis of microflora associated with dentoalveolar abscesses. *J Clin Microbiol* 34:3537-3542.

- Edwardsson S (1974). Bacteriological studies on deep areas of carious dentine. *Odontol Revy* 25(Suppl 32):1-143.
- Edwardsson S (1987). Bacteriology of dentin caries. In: Dentine and dentine reactions in the oral cavity. Thylstrup A, Leach SA, Qvist V, editors. Oxford: IRL Press Ltd., pp. 95-102.
- Eide B, Lie T, Selvig KA (1984). Surface coatings on dental cementum incident to periodontal disease. II. Scanning electron microscope confirmation of a mineralized cuticle. *J Clin Periodontol* 11:565-575.
- Elder BL, Fives-Taylor P (1986). Characterization of monoclonal antibodies specific for adhesion: isolation of adhesin of *Streptococcus sanguis* FW213. *Infect Immun* 54:421-427.
- Fabricius L, Dahlén G, Öhman A, Möller ÅJR (1982a). Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *Scand J Dent Res* 90:134-144.
- Fabricius L, Dahlén G, Holm SE, Möller ÅJR (1982b). Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand J Dent Res* 90:200-206.
- Garberoglio R, Brännström M (1976). Scanning electron microscopic investigation of human dentinal tubules. *Arch Oral Bio* 21:355-362.
- Gibbons RJ (1984). Microbial ecology: adherent interactions which may affect microbial ecology in the mouth. *J Dent Res* 63:378-385.
- Gibbons RJ (1989). Bacterial adhesion to oral tissues: a model for infectious diseases. *J Dent Res* 68:750-760.
- Gillaspy AF, Patti JM, Smeltzer MS (1997). Transcriptional recognition of the *Staphylococcus aureus* collagen adhesin gene *cna*. *Infect Immun* 65:1536-1540.
- Giuliana G, Ammatuna P, Pizzo G, Capone F, D'Angelo M (1997). Occurrence of invading bacteria in radicular dentin of periodontally diseased teeth: microbiological findings. *J Clin Periodontol* 24:478-485.
- Goulbourne PA, Ellen RP (1991). Evidence that *Porphyromonas (Bacteroides) gingivalis* fimbriae function in adhesion to *Actinomyces viscosus*. *J Bacteriol* 173:5266-5274.
- Haapasalo M (1989). *Bacteroides* spp. in dental root canal infections. *Endod Dent Traumatol* 5:1-10.
- Haapasalo M, Ørstavik D (1987). *In vitro* infection and disinfection of dentinal tubules. *J Dent Res* 66:1375-1379.
- Hahn C-L, Overton B (1997). The effects of immunoglobulins on the convective permeability of human dentine *in vitro*. *Arch Oral Biol* 42:835-843.
- Hahn C-L, Falkler WA Jr, Minah GE (1990). Microbiological studies of carious dentine from human teeth with irreversible pulpitis. *Arch Oral Biol* 36:147-153.
- Hartzell T (1911). The practical surgery of the root surface in pyorrhea. *Dent Cosmos* 53:513-521.
- Hashioka K, Yamasaki M, Nakane A, Horiba N, Nakamura H (1992). The relationship between clinical symptoms and anaerobic bacteria from infected root canals. *J Endodont* 18:558-561.
- Hasty DL, Ofek I, Courtney HS, Doyle RJ (1992). Multiple adhesins of streptococci. *Infect Immun* 60:2147-2152.
- Hill PE, Knox KW, Schamschula RG, Tabua J (1977). The identification and enumeration of actinomyces from plaque of New Guinea indigenes. *Caries Res* 11:327-335.
- Hoshino E (1985). Predominant obligate anaerobes in human carious dentin. *J Dent Res* 64:1195-1198.
- Isogai H, Isogai E, Yoshimura F, Suzuki T, Kagota W, Takano K (1988). Specific inhibition of adherence of an oral strain of *Bacteroides gingivalis* 381 to epithelial cells by monoclonal antibodies against the bacterial fimbriae. *Arch Oral Biol* 33:479-485.
- Izumi T, Yamada K, Inoue H, Watanabe K, Nishigawa Y (1998). Fibrinogen/fibrin and fibronectin in the dentin-pulp complex after cavity preparation in rat molars. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 86:587-591.
- Jenkinson HF (1994). Cell surface protein receptors. *FEMS Microbiol Lett* 121:133-140.
- Jenkinson HF, Demuth DR (1997). Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. *Molec Microbiol* 23:183-190.
- Jenkinson HF, Lamont RJ (1997). Streptococcal adhesion and colonization. *Crit Rev Oral Biol Med* 8:175-200.
- Jontell M, Okiji T, Dahlgren U, Bergenholtz G (1998). Immune defense mechanisms of the dental pulp. *Crit Rev Oral Biol Med* 9:179-200.
- Jung I-Y, Choi B-k, Kum K-Y, Roh B-D, Lee S-J, Lee C-Y, et al. (2000). Molecular epidemiology and association of putative pathogens in root canal infection. *J Endodont* 26:599-604.
- Kakehashi S, Stanley HR, Fitzgerald RJ (1965). The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* 20:340-349.
- Kantz WE, Henry CA (1974). Isolation and classification of anaerobic bacteria from intact pulp chambers of non-vital teeth in man. *Arch Oral Biol* 19:91-96.
- Keyes PH (1960). The infectious and transmissible nature of experimental caries. Findings and implications. *Arch Oral Biol* 1:304-320.
- Knutsson G, Jontell M, Bergenholtz G (1994). Determination of plasma proteins in dentinal fluid from cavities prepared in healthy young human teeth. *Arch Oral Biol* 39:185-190.
- Kolenbrander PE (2000). Oral microbial communities: biofilms, interactions, and genetic systems. *Annu Rev Microbiol* 54:413-437.
- Kolenbrander PE, London J (1993). Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 175:3247-3253.
- Kopczyk R, Conroy C (1968). The attachment of calculus to root planed surfaces. *Periodontics* 6:78-83.
- Lamont RJ, Rosan B, Murphy GM, Baker CT (1988a). *Streptococcus sanguis* surface antigens and their interactions with saliva. *Infect Immun* 56:64-70.
- Lamont RJ, Rosan B, Baker CT, Nelson GM (1988b). Characterization of an adhesin antigen of *Streptococcus sanguis* G9B. *Infect Immun* 56:2417-2423.
- Lamont RJ, Hersey SG, Rosan B (1992). Characterization of the adherence of *Porphyromonas gingivalis* to oral streptococci. *Oral Microbiol Immunol* 7:193-197.
- Lamont RJ, Chan A, Belton M, Izutso KT, Vasel D, Weinberg A (1995). *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 63:3878-3885.
- Lana MA, Ribeiro-Sobrinho AP, Stehling R, Garcia GD, Silva BKC, Hamdan JS, et al. (2001). Microorganisms isolated from root canals presenting necrotic pulp and their drug susceptibility *in vitro*. *Oral Microbiol Immunol* 16:100-105.
- Langeland K, Rodrigues H, Dowden W (1974). Periodontal disease, bacteria and pulpal histopathology. *Oral Surg* 37:257-270.
- Lawry J, Switalski LM (1996). Cloning and expression of collagen adhesin of *Streptococcus mutans* (abstract). *J Dent Res* 75(Spec Iss):96.
- Le Goff A, Bunetel L, Mouton C, Bonnaure-Mallet M (1997). Evaluation of root canal bacteria and their antimicrobial susceptibility in teeth with necrotic pulp. *Oral Microbiol Immunol* 12:318-322.
- Li J, Ellen RP, Hoover CL, Felton JR (1991). Association of proteases of *Porphyromonas (Bacteroides) gingivalis* with its adhesion to *Actinomyces viscosus*. *J Dent Res* 70:82-86.
- Li Y, Caufield PW (1998). Arbitrarily primed polymerase chain reaction fingerprinting for the genotypic identification of mutans streptococci from humans. *Oral Microbiol Immunol* 13:17-22.

- Linde A, Goldberg M (1993). Dentinogenesis. *Crit Rev Oral Biol Med* 45:679-728.
- Liu T, Gibbons RJ (1990). Binding of streptococci of the mutans group to type 1 collagen associated with apatitic surfaces. *Oral Microbiol Immunol* 5:131-136.
- Liu T, Gibbons RJ, Hay DI (1990). *Streptococcus cricetus* and *Streptococcus rattus* bind different segments of collagen molecules. *Oral Microbiol Immunol* 5:143-148.
- Loesche WJ, Syed SA (1973). The predominant cultivable flora of carious plaque and carious dentin. *Caries Res* 7:201-216.
- Love RM (1996a). Bacterial penetration of the root canal of intact incisor teeth after a simulated traumatic injury. *Endod Dent Traumatol* 12:289-293.
- Love RM (1996b). Regional variation in root dentinal tubule infection by *Streptococcus gordonii*. *J Endodont* 22:290-293.
- Love RM (1997). Effects of dental trauma on the pulp. *Pract Perio Aesthet Dent* 9:427-436.
- Love RM (2001). *Enterococcus faecalis*—a mechanism for its role in endodontic failure. *Int Endodont J* 34:399-405.
- Love RM, Chandler NP, Jenkinson HF (1996). Penetration of smeared or nonsmeared dentine by *Streptococcus gordonii*. *Int Endodont J* 29:2-12.
- Love RM, McMillan MD, Jenkinson HF (1997). Invasion of dentinal tubules by oral streptococci is associated with collagen recognition mediated by the antigen I/II family of polypeptides. *Infect Immun* 65:5157-5164.
- Love RM, McMillan MD, Park Y, Jenkinson HF (2000). Coinvasion of dentinal tubules by *Porphyromonas gingivalis* and *Streptococcus gordonii* depends upon binding specificity of streptococcal antigen I/II adhesin. *Infect Immun* 68:1359-1365.
- Lundy T, Stanley HR (1969). Correlation of pulpal histopathology and clinical symptoms in human teeth subjected to experimental irritation. *Oral Surg* 27:187-201.
- Maita E, Simpson MD, Tao L, Pashley DH (1991). Fluid and protein flux across the pulp-dentin complex of the dog in vivo. *Arch Oral Biol* 36:103-110.
- Malamud D (1985). Influence of salivary proteins on the fate of oral bacteria. In: Molecular basis of oral microbial adhesion. Mergenhagen SE, Rosan B, editors. Washington, DC: American Society for Microbiology, pp. 117-124.
- Marshall GW (1993). Dentin microstructure and characterization. *Quintessence Int* 24:606-617.
- Massey WLK, Romberg DM, Hunter N, Hume WR (1993). The association of carious dentin microflora with tissue changes in human pulpitis. *Oral Microbiol Immunol* 8:30-35.
- Mejäre B, Mejäre I, Edwardsson S (1979). Bacteria beneath composite restorations—a culturing and histobacteriological study. *Acta Odontol Scand* 37:267-275.
- Mejäre B, Mejäre I, Edwardsson S (1987). Acid etching and composite resin restorations. A culturing and histologic study on bacterial penetration. *Endod Dent Traumatol* 3:1-5.
- Meryon SD, Brook AM (1990). Penetration of dentine by three oral bacteria *in vitro* and their associated cytotoxicity. *Int Endodont J* 23:196-202.
- Meryon SD, Jakeman KJ, Browne RM (1986). Penetration *in vitro* of human and ferret dentine by three bacterial species in relation to their potential role in pulpal inflammation. *Int Endodont J* 19:213-220.
- Micheli VJ, Schuster GS, Pashley DH (1980). Bacterial penetration of human dentin *in vitro*. *J Dent Res* 59:1398-1403.
- Miller WD (1890). The micro-organisms of the human mouth. Basel: S. Karger, 1973.
- Möller ÅJR, Fabricius L, Dahlén G, Öhman AE, Heyden G (1981). Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scand J Dent Res* 89:475-484.
- Moore WEC, Moore LVH (1994). The bacteria of periodontal diseases. *Periodontology* 2000 5:66-77.
- Moskow B (1969). Calculus attachment in cemental separations. *J Periodontol* 40:125-130.
- Murray PA, Levine MJ, Reddy MS, Tabak LA, Bergey EJ (1986). Preparation of a sialic acid-binding protein from *Streptococcus mitis* KS32Ar. *Infect Immun* 53:359-365.
- Nagaoka S, Liu H-J, Minemoto K, Kawagoe M (1995). Microbial induction of dentinal caries in human teeth *in vitro*. *J Endodont* 21:546-551.
- Naito Y, Gibbons RJ (1988). Attachment of *Bacteroides gingivalis* to collagenous substrata. *J Dent Res* 67:1075-1080.
- Naito Y, Tohada H, Okuda K, Takazoe I (1993). Adherence and hydrophobicity of invasive and noninvasive strains of *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 8:195-202.
- Okuda K, Slots J, Genco RJ (1981). *Bacteroides gingivalis*, *Bacteroides asaccharolyticus*, and *Bacteroides melaninogenicus* sub-species: cell surface morphology and adherence to erythrocytes and human buccal epithelial cells. *Curr Microbiol* 6:7-12.
- Olgart L, Brännström M, Johnson G (1974). Invasion of bacteria into dentinal tubules. Experiments *in vivo* and *in vitro*. *Acta Odontol Scand* 32:61-70.
- Oligino L, Fives-Taylor P (1993). Overexpression and purification of a fimbria-associated adhesin of *Streptococcus parasanguis*. *Infect Immun* 61:1016-1022.
- Ørstavik D, Haapasalo M (1990). Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod Dent Traumatol* 6:142-149.
- Ozaki K, Matsua T, Nakae H, Noiri Y, Yoshiyama M, Ebisu S (1994). A quantitative comparison of selected bacteria in human carious dentine by microscopic counts. *Caries Res* 28:137-145.
- Pashley DH (1990). Clinical considerations in microleakage. *J Endodont* 16:70-77.
- Pashley DH (1992). Dentin permeability and dentin sensitivity. *Proc Finn Dent Soc* 88(Suppl 1):215-224.
- Pashley DH (1996). Dynamics of the pulpo-dentin complex. *Crit Rev Oral Biol Med* 7:104-133.
- Pashley DH, Nelson R, Kepler E (1982). The effects of plasma and salivary constituents on dentin permeability. *J Dent Res* 61:978-981.
- Patti JM, Bremell T, Krajewska-Pietrasik D, Abdelnour A, Tarkowski A, Rydén C, et al. (1994). The *Staphylococcus aureus* collagen adhesin is a virulence determinant in septic arthritis. *Infect Immun* 62:152-161.
- Perez F, Rochd T, Lodter J-P, Calas P, Michel G (1993). *In vitro* study of the penetration of three bacterial strains into root dentine. *Oral Surg Oral Med Oral Pathol* 76:97-103.
- Peters LB, Wesselink PR, Moorer WR (1995). The fate and role of bacteria left in root dentinal tubules. *Int Endodont J* 28:95-99.
- Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ (2001). Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J Endodont* 27:76-81.
- Ray HA, Trope M (1995). Periapical status of endodontically treated teeth in relation to the technical quality of the root filling and the coronal restoration. *Int Endodont J* 28:12-18.
- Rôças IN, Siqueira JF Jr, Santos KRN, Coelho AMA (2001). "Red complex" (*Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Treponema denticola*) in endodontic infections: a molecular approach. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 91:468-471.
- Sandros J, Papapanou PN, Nannmark U, Dahlén G (1994). *Porphyromonas gingivalis* invades human pocket epithelium *in vitro*. *J Periodontal Res* 29:62-9.
- Scannapieco FA (1994). Saliva-bacterium interactions in oral microbial ecology. *Crit Rev Oral Biol Med* 5:203-248.
- Schenkels LCPM, Ligtenberg AJM, Veerman ECI, Nieuw

- Amerongen AV (1993). Interaction of salivary glycoprotein EP-GP with the bacterium *Streptococcus salivarius* HB. *J Dent Res* 72:1559-1565.
- Shovelton DH (1964). The presence and distribution of microorganisms within non-vital teeth. *Br Dent J* 117:101-107.
- Siqueira JF, De Uzeda M, Fonseca MEF (1996). A scanning electron microscopic evaluation of *in vitro* dentinal tubules penetration by selected anaerobic bacteria. *J Endodont* 22:308-310.
- Smith DJ, Akita H, King WF, Taubman MA (1994). Purification and antigenicity of a novel glucan-binding protein of *Streptococcus mutans*. *Infect Immun* 62:2545-2552.
- Socransky SS, Haffajee AD (1992). The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 63:322-331.
- Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134-144.
- Sun J-W, Wanda S-Y, Camilli A, Curtiss R III (1994). Cloning and DNA sequencing of the dextranase inhibitor gene (dei) from *Streptococcus sobrinus*. *J Bacteriol* 176:7213-7222.
- Sundqvist G (1976). Bacteriological studies of necrotic dental pulps. Umeå, Sweden: University of Umeå.
- Sundqvist G (1992a). Associations between microbial species in dental root canal infections. *Oral Microbiol Immunol* 7:257-262.
- Sundqvist G (1992b). Ecology of the root canal flora. *J Endodont* 18:427-430.
- Sundqvist G (1994). Taxonomy, ecology, and pathogenicity of the root canal flora. *Oral Surg Oral Med Oral Pathol* 78:522-530.
- Sundqvist G, Johansson E, Sjögren U (1989). Prevalence of black-pigmented Bacteriodes species in root canal infections. *J Endodont* 15:13-19.
- Switalski LM, Butcher WG, Caufield PC, Lantz MS (1993). Collagen mediates adhesion of *Streptococcus mutans* to human dentin. *Infect Immun* 61:4119-4125.
- Syed SA, Loesche WJ, Pape HL, Grenier E (1975). Predominant cultivable flora isolated from human root surface caries plaque. *Infect Immun* 11:727-731.
- Takahashi Y, Sandberg AL, Ruhl S, Muller J, Cisar JO (1997). A specific cell surface antigen of *Streptococcus gordonii* is associated with bacterial hemagglutination and adhesion to α 2-3-linked sialic acid-containing receptors. *Infect Immun* 65:5042-5051.
- Terpenning M, Bretz W, Lopatin D, Langmore S, Dominguez B, Loesche W (1993). Bacterial colonization of saliva and plaque in the elderly. *Clin Infect Dis* 16:S314-S316.
- Tronstad L (1973). Ultrastructural observations on human coronal dentine. *Scand J Dent Res* 81:101-111.
- Tronstad L, Langeland K (1971). Effect of attrition on subjacent dentin and pulp. *J Dent Res* 50:17-30.
- Valderhaug J (1974). A histologic study of experimentally induced periapical inflammation in primary teeth in monkeys. *Int J Oral Surg* 3:111-123.
- Van der Mei HC, Cox SG, Geertsema-Doornbusch GI, Doyle RJ, Busscher HJ (1993). A critical appraisal of positive cooperativity in oral streptococcal adhesion: Scatchard analyses of adhesion data versus analyses of the spatial arrangement of adhering bacteria. *J Gen Microbiol* 139:937-948.
- van Houte J (1994). Role of microorganisms in caries etiology. *J Dent Res* 73:672-681.
- van Houte J, Lopman J, Kent R (1994). The predominant cultivable flora of sound and carious human root surfaces. *J Dent Res* 73:1727-1734.
- Visai L, Bozzini S, Raucci G, Toniolo A, Speziale P (1995). Isolation and characterization of a novel collagen-binding protein from *Streptococcus pyogenes* strain 6414. *J Biol Chem* 270:347-353.
- Vojinovic O, Nyborg H, Brännström M (1973). Acid treatment of cavities under resin fillings: bacterial growth in dentinal tubules and pulpal reactions. *J Dent Res* 52:1189-1193.
- Vongsavan N, Matthews B (1994). The relation between fluid flow through dentine and the discharge of intradental nerves. *Arch Oral Biol* 39:140S.
- Weinberg A, Belton CM, Park Y, Lamont RJ (1997). Role of fimbriae in *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 65:313-316.
- Wittgow WC, Sabiston CB (1975). Microorganisms from pulpal chambers of intact teeth with necrotic pulps. *J Endodont* 1:168-171.
- Wu H, Fives-Taylor PM (1999). Identification of dipeptide repeats and a cell wall sorting signal in the fimbriae-associated adhesin, Fap1, of *Streptococcus parasanguis*. *Mol Microbiol* 34:1070-1081.
- Yao ES, Lamont RJ, Leu SP, Weinberg A (1996). Interbacterial binding among pathogenic and commensal bacteria. *Oral Microbiol Immunol* 11:35-41.