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Bacterial Pathogenesis and Mediators in Apical Periodontitis

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Apical periodontitis is a group of inflammatory diseases caused by microorganisms (mainly bacteria) infecting the necrotic root canal system. The pathogenesis of different types of apical periodontitis and even the same type in different individuals is unlikely to follow a stereotyped fashion with regard to the involved bacterial mediators. Disease pathogenesis is rather complex and involves a multitude of bacteria- and host-related factors. This review article discusses the bacterial pathogenesis of acute and chronic apical periodontitis, with the main focus on the bacterial mediators conceivably involved in the different stages of the infectious process, including secreted products (enzymes, exotoxins, N-formyl-methionyl-leucyl-phenylalanine peptides, heat-shock proteins and metabolic end-products) and structural components (lipopolysaccharide, peptidoglycan, lipoteichoic acid, lipoproteins, fimbriae, flagella, outer membrane proteins and vesicles, DNA, and exopolysaccharides). Knowledge of the bacterial factors involved in the pathogenesis of apical periodontitis is important to the understanding of the disease process and to help establishing proper therapeutic measures to inactivate this bacterial "artillery".

Key Words: endodontic infection, bacterial pathogenicity, virulence factors, apical periodontitis.

INTRODUCTION

Apical periodontitis is a group of inflammatory diseases caused by microorganisms (mainly bacteria) infecting the necrotic root canal system. The process starts after pulp necrosis as a result of caries, trauma or iatrogenic procedures, when bacteria invade and colonize the root canal system. As a consequence of necrosis, the endodontic environment becomes a selective habitat for the establishment of a mixed microbiota conspicuously dominated by anaerobic bacteria (1). In late stages of the infectious process, bacterial organizations resembling biofilms can be observed adhered to the canal walls (2-4). Thus, there is a current trend to consider apical periodontitis as a biofilm-induced disease. Bacteria colonizing the necrotic root canal come into contact with the periodontal ligament via apical or lateral foramens, induce damage and give rise to inflammatory changes. The host defenses, in turn, can eliminate bacteria egressing from the canal, but are unable to eradicate bacteria entrenched in the sanctuary of the necrotic root canal, which lacks an active microcirculation and is consequently beyond the reaches of body defenses. Disease pathogenesis is rather complex and involves a multitude of bacteria- and host-related factors. This review article discusses the bacterial pathogenesis of apical periodontitis, with the main focus on the bacterial mediators (or virulence factors) involved in the different stages of the disease process.

ENDODONTIC PATHOGENS AND MECHANISMS OF PATHOGENICITY

Bacteria involved in the pathogenesis of apical periodontitis may have participated in the early stages of pulp inflammation and necrosis or they may have gained entry into the canal space any time after pulpal necrosis. In the former situation, involved bacteria are usually those present in the advanced front of caries lesions and from saliva bathing the affected area. Bacteria impli-

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cated in pulp disease first adhere to the dentinal walls and colonize this surface, forming authentic biofilms. Diffusion of bacterial products through dentinal tubules induces pulpal inflammation long before the tissue is exposed. After pulp exposure, the surface of the tissue can also be colonized and covered by bacteria present in the caries biofilm. The exposed pulp tissue is in direct contact with bacteria and their products, and responds with severe inflammation. Some tissue invasion by some bacteria may also occur. Bacteria in the battle front have to survive the attack from the host defenses and at the same time have to acquire nutrients to keep themselves alive. In this bacteria-pulp clash, the latter invariably is "defeated" and becomes necrotic. Therefore, bacteria move forward and "occupy the territory", i.e., colonize the necrotic tissue. These events occur *per* tissue compartments, which coalesce and move towards the apical part of the canal until virtually the entire root canal is necrotic and infected. At this stage, involved bacteria can be regarded as the early root canal colonizers or pioneer species.

Bacteria colonizing the necrotic root canal start inducing damage to the periradicular tissues and give rise to inflammatory changes. In fact, periradicular inflammation can be observed even before the entire root canal is necrotic (5-7). Therefore, the early colonizers play an important role in the initiation of the apical periodontitis disease process. The environmental conditions in the canal are modified by pioneer species and the disease process, and may now be conducive to the establishment of bacterial groups different from the early colonizers. Once the pulp is necrotic, species other than those that participated in the initial infectious process may also have access to the canal via coronal exposure or exposed dentinal tubules. In fact, a shift in the microbiota is observed and is resultant of rearrangements in the proportions of the pioneer species and arrival of latecomers. Some early colonizers are expected to no longer participate in the consortium in advanced disease. As time passes, the endodontic microbiota becomes more and more structurally and spatially organized. Some virulence attributes required for pathogens to thrive in other sites may be of no value for bacteria that reach the root canal after necrosis as, for instance, the ability to evade the host defenses. This is because latecomers face no significant opposition from host defenses, which are no longer active in the canal after necrosis. Although colonization may appear an easy task for late colonizers, other environmental factors, such as interaction with pioneer species, oxygen tension and nutrients availability, will determine whether the new species entering the canal will succeed in establishing therein. Thus, latecomers will join the early colonizers to make up a dynamic mixed community in the root canal. Ultimately, the root canals of teeth evincing radiographically detectable apical periodontitis lesions harbor early colonizers that managed to stay in the canals and late colonizers that managed to adapt to the new, but propitious, environmental conditions.

The usual sequential stages for bacterial infections in several body sites include a) attachment to and colonization of host surfaces; b) invasion of host tissues; c) survival within the tissue by acquiring nutrients and evading host defense mechanisms; d) and induction of direct or indirect damage to the host tissues. The process of early pulp infection is likely to follow similar events, even though it should be assumed that the sequence of events is more didactic than real and some phases may overlap or swap positions. Several bacterial mediators (virulence factors) are involved in each of these events.

Bacteria exert their pathogenicity by wreaking havoc to the host tissues through direct and/or indirect mechanisms. Bacterial factors that cause direct tissue harm include those that damage host cells and/or the intercellular matrix of the connective tissue. These factors usually involve secreted products, including enzymes, exotoxins and metabolic end-products (8). Furthermore, bacterial structural components, including peptidoglycan, lipoteichoic acid, fimbriae, flagella, outer membrane proteins and vesicles, DNA, exopolysaccharides and lipopolysaccharide, are shed into the periradicular tissues and act as modulins by stimulating the development of host immune reactions capable not only of defending the host against infection but also of causing severe tissue destruction (9, 10). For instance, inflammatory and non-inflammatory host cells can be stimulated by bacterial components to release chemical mediators such as cytokines and prostaglandins, which are involved in the induction of bone resorption characteristically observed in chronic apical periodontitis lesions (11). Pro-inflammatory cytokines stimulate osteoclastic bone resorption by either enhancing the proliferation and differentiation of osteoclast precursors or promoting activation of mature osteoclasts, or both (12). Another example of indirect dam-

age caused by bacteria is the formation of purulent exudate in acute apical abscesses. Host defense mechanisms against bacteria emanating from the root canal appear to be the most important factor involved in pus formation associated with abscesses. Formation of oxygen-derived free radicals, such as superoxide and hydrogen peroxide, alongside the release of lysosomal enzymes by polymorphonuclear leukocytes, gives rise to destruction of the connective extracellular matrix, leading to pus formation (13). Therefore, bacteria can exert indirect destructive effects, which seem to be even more significant in the tissue damage associated with acute and chronic apical periodontitis lesions.

Depending on several factors, apical periodontitis can be chronic or acute. A chronic disease is usually associated with low virulence of the involved bacterial consortium, which though represents a persistent source of aggression to the tissues. Persistence can be related to community organization in biofilms and inaccessibility to host defenses because of the anatomic location of the infection. An acute infection, in turn, is usually caused by a high virulent bacterial consortium. Such high virulence may be due to the presence of virulent species or strains and/or the occurrence of synergism between species. Acute infections are usually related to bacterial cells in a planktonic state, at high numbers, with tissue invasion ability, counteracted by a diminished host resistance. It has been demonstrated for some pathogens that genes coding for many virulence factors are much more highly expressed in planktonic cells than in biofilm cells, suggesting that planktonic cells are more likely to participate in acute infections (14).

The isolate location of the root canal microbiota indicates that to exert its pathogenicity the bacteria must either invade the periradicular tissues or their products and/or structural components must penetrate the tissue and be able to evoke a defense response in the host. Bacteria can invade tissues either by their motility or by growth. Motile bacteria can escape phagocytes by a rapid movement. Invasion by growth requires that the rate of reproduction overcomes the host defense mechanisms. Frank invasion of the periradicular tissues is rather uncommon and, when it occurs, bacteria are usually quickly eliminated. In some instances, which will depend upon several factors, massive invasion of the periradicular tissues by bacteria may result in abscess formation. The presence of more virulent species or strains, or a more virulent mixed consortium, can predispose to abscess formation. However, it is assumed that the oral microbiota contains only a few pathogenic species, and most of them have low virulence. This is consistent with the chronic slowly progressive nature of the most common form of apical periodontitis. Therefore, as bacterial infection (invasion, survival and proliferation) of the periradicular tissues represent a rare occurrence, except for abscess cases, direct or indirect aggression to the tissues is caused by bacterial secreted products or structural components that diffuse out from the canal or are released by bacterial cells that reached the periradicular tissues but are quickly destroyed therein by the host defenses. In acute cases or in the rare cases where an asymptomatic lesion is infected (e.g., actinomycosis, infected cysts), tissue damage comes also from factors released by viable bacterial cells directly in the tissues.

More than likely, few, if any, of the putative endodontic pathogens are individually capable of inducing all of the events involved in the pathogenesis of the different forms of apical periodontitis. Probably, the process requires an integrated and orchestrated interaction of the selected members of the mixed endodontic microbiota and their respective virulence attributes.

BACTERIAL VIRULENCE FACTORS

Bacterial virulence factors comprise structural cellular components and released products. Bacterial strategies that contribute to pathogenicity, such as the ability to co-aggregate and form biofilms, have also been regarded as virulence factors, but will not be discussed herein. Most bacterial virulence factors have their primary functions other than causing host tissue damage. They have a structural or physiological role and that of a virulence factor is merely coincidental and consequential. Different virulence factors usually act in combination at various stages of infection, and a single factor may have several functions at different stages. Virulence factors are involved in every step of the infectious process (attachment, invasion, survival, and damage) (Table 1).

Structural Components

Lipopolysaccharide (LPS)

Richard Pfeiffer, one of the Robert Koch's

students, observed that *Vibrio cholerae* produced not only a heat-labile exotoxin, but also a heat-resistant substance only released after cell death (15). This substance was named endotoxin, which was subsequently revealed to be a misnomer, since endotoxins reside on the surface of bacteria, not inside. Even though the terms lipopolysaccharide (LPS) and endotoxin have been used interchangeably, it has been recommended that the term LPS be reserved for purified bacterial extracts that are free of contaminants (mainly protein) and the term endotoxin be used to refer to macromolecular complexes of LPS, protein and phospholipids.

The LPS molecule consists of a hydrophilic polysaccharide, subdivided into the O-polysaccharide specific chain (O-antigen) and the core oligosaccharide, and a hydrophobic glycolipid component, termed lipid A (16). Lipid A is found embedded in the outer membrane of the Gram-negative cell wall, while the core and the O-antigen portions extend outward from the bacterial surface. The long polysaccharide chains of LPS can allow the fixation of the complement system at a site distant from the bacterial cell membrane, protecting the bacterium from the lethal lytic effect of that host defense system. Except for activating the complement system through the alternative pathway with consequent release of pro-inflammatory by-products of the system, the LPS molecule is virtually not toxic when it is incorporated into the bacterial outer membrane. Lipid A is set free from the outer membrane during bacterial multiplication or after death, when LPS is released either as a free form, or as a complex of LPS with bacterial surface proteins (endotoxin). As a consequence of release, lipid A is then exposed to host cells and can give rise to a full range of biologic events.

The macrophage appears to be a key cell involved in host response to LPS. After release from bacteria, LPS is initially bound to a plasm protein called LPS-binding protein (LBP) and is then delivered to CD14, a cell receptor for LPS on the surface of macrophages (17). Subsequent activation of the macrophage is a result of signal triggered by a signal-transducing receptor called Toll-like receptor (TLR). The Toll family

Table 1. Bacterial virulence factors involved in different stages of the infectious process.

Function	Virulence factors
Attachment	Adhesins (fimbriae, afimbrial surface proteins) Exopolysaccharides Lipoteichoic acid Outer membrane proteins Outer membrane vesicles
Invasion	Flagella Enzymes (collagenase, hyaluronidase, chondroitin sulfatase, fibrinolysin, acid phosphatase, and Dnase)
Survival (evasion of host defenses or acquisition of nutrients)	Exopolysaccharides (capsule) IgA, IgG, IgM, C3, and C5 proteinases Lipopolysaccharide (antigen-O portion) Flagella Exotoxins Heat-shock proteins Metabolic end-products
Direct damage	Exotoxins Enzymes (collagenase, hyaluronidase, chondroitin sulfatase, gingipains, aminopeptidases, phospholipase, neuraminidase, and acid phosphatase) Metabolic end-products (short-chain fatty acids, polyamines, volatile sulfur compounds, indole, ammonia)
Indirect damage	Lipopolysaccharide (mainly lipid A portion) Peptidoglycan Lipoteichoic acid Fimbriae Exopolysaccharides Outer membrane proteins (porins) Lipoproteins DNA Heat-shock proteins

of receptors encompasses transmembrane molecules linking the extracellular compartment, where contact and recognition of pathogens occurs, and the intracellular compartment, where signaling cascades leading to cellular responses are initiated. TLRs are responsible for cell signaling to a variety of bacterial components (Table 2). TLR-4 is involved in cellular activation by LPS from most bacteria. However, TLR-2 may be involved in cell signalling to some types of LPS, such as that from *Porphyromonas gingivalis*. Engagement of the receptor activates transcription factors, which induce activation of genes encoding several cytokines.

The biologic effects of LPS include:

a) Activation of macrophages/monocytes with consequent synthesis and release of pro-inflammatory cytokines (IL-1β, IL-6, CXCL8 or IL-8, TNF-α), prostaglandins, nitric oxide, and oxygen-derived free radicals (9,10,18). These substances are chemical mediators of inflammation and most of them can stimulate bone resorption;

b) Activation of the complement system. Some products of complement activation are chemotactic to inflammatory cells (C5a), act as opsonins (C3b), and can increase vascular permeability (C3a and C5a).

c) Activation of the Hageman factor (19-21), the first step of the intrinsic clotting system, triggering the coagulation cascade or the production of bradykinin, an important chemical mediator of inflammation;

d) Induction of the expression of leukocyte adhesion molecules on endothelial cells (22-25), which are important in the early stages of inflammation;

e) Stimulation of osteoclast differentiation and bone resorption, particularly via interactions with TLR-4 on osteoblast-lineage cells (26). LPS induces RANKL expression in osteoblasts and stimulates these cells to secrete interleukin (IL)-1, IL-6, prostaglandin E_2 (PGE_2), and TNF-α, each known to induce osteoclast activity and differentiation (12,27,28).

f) LPS may be mitogenic to B lymphocytes and epithelial cells (29).

g) LPS can stimulate naive B cells in the absence of T-cell help. At low concentrations, LPS stimulates specific antibody production. At high concentrations, this molecule can cause nonspecific polyclonal activation of B cells (30).

h) It has been recently demonstrated that trigeminal afferent neurons express the TLR4 and CD14 receptor complex and that LPS activation of TLR4/CD14 may trigger intracellular signaling cascades, leading to peripheral release of neuropeptides and central nociceptive neurotransmission (31). This raises the possibility that one of the mechanisms of pain associated with bacterial infectious processes can result from direct effects of LPS on sensory fibers via interaction and direct activation of the TLR4/CD14 complex.

The concentration of LPS in infected canals is obviously expected to be directly proportional to the load (number of cells) of Gram-negative bacteria (32). Studies have revealed that the content of endotoxin or LPS in infected root canals is higher in teeth with symptomatic apical periodontitis, teeth with periradicular bone destruction, or teeth with persistent exudation than in those without them (32-36). Murakami et al. (37) detected *Porphyromonas endodontalis* LPS in samples from infected root canals or in the pus samples of acute abscesses of about 90% of the patients tested. They suggested that *P. endodontalis* LPS can play an integral role as a potent stimulator of inflammatory cytokines that are involved in the formation of acute abscesses (37). Dahlén et al. (38) inoculated *Fusobacterium nucleatum* LPS into the root canals of monkeys and reported the occurrence of inflammatory reaction in the periradicular tissues of all the experimental teeth with resorption of both bone and teeth. Dwyer and Torabinejad (39) evaluated histologically and radiographically the periradicular tissues of cats after deposition of *E. coli* endotoxin solutions in the root canals and concluded

Table 2. Microbial ligands recognized by Toll-like family members.

TLRs	Ligands	Source
TLR2	Peptidoglycan	Gram-positive and Gram-negative species
	Lipoteichoic acid	Gram-positive species
	Lipoproteins	Gram-negative species
	LPS	*Porphyromonas gingivalis* and *Leptospira interrogans*
TLR4	LPS	Most Gram-negative species
TLR5	Flagellin	Flagellated Gram-positive and Gram-negative species
TLR9	CpG motifs of bacterial DNA	All bacteria

that endotoxin may have a role in the induction and perpetuation of periradicular inflammatory lesions. Such results were similar to those obtained by Pitts et al. (40) after inoculation of *Salmonella minnesota* endotoxin solution in the root canals of dogs.

Not all Gram-negative bacteria produce LPS. For instance, some treponemes possess lipooligosaccharides (with a carbohydrate portion much shorter), and lipoproteins in the outer membrane. Treponemal lipooligosaccharides and lipoproteins may have similar bioactivity to LPS (41-44).

Peptidoglycan

Except for cell wall-less mycoplasmas, every bacterium contains peptidoglycan in its cell wall, whose primary function seems to be protecting the cell against osmotic lysis. Peptidoglycan is a complex polymer consisting of two parts: a glycan portion and a tetrapeptide portion. Due to cross-linkages, peptidoglycan forms a strong, multilayered sheet that entirely surrounds the bacterial cell. In Gram-positive bacteria, there are as many as 40-100 sheets of peptidoglycan, comprising up to 50% of cell wall material. In Gram-negative bacteria, there appears to be only one or two sheets, comprising 5-10% of the cell wall material. Peptidoglycan may induce diverse biological effects (45), which may play a role in the pathogenesis of apical periodontitis lesions. These effects include activation of macrophages/monocytes with consequent release of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and G-CSF, and activation of the complement system via the alternative pathway (10,45,46). Signalling of peptidoglycan is mediated mainly through TLR-2 (47).

Lipoteichoic acid

Anionic polymers, such as the lipoteichoic acid (LTA), are major components of the cell wall of Gram-positive bacteria, accounting for up to 50% of its dry weight. LTA is a polymer of glycerol phosphate covalently attached to a glycolipid in the cytoplasmic membrane and protruding through the peptidoglycan layer. LTA can activate macrophages/monocytes and induce the release of pro-inflammatory cytokines, such as IL-1β, IL-6, CXCL8 and TNF-α (10,46). LTA exerts its effects by signaling via TLR-2 (47). LTA may also activate the complement system (48). All these effects may indirectly account for the induction of tissue damage. Because LTA resembles LPS in certain biologic effects, it can be considered as the Gram-positive counterpart of LPS.

Outer Membrane Proteins

Approximately 50% of the dry mass of the outer membrane of Gram-negative bacteria consists of proteins. Apart from their structural role, outer membrane proteins (OMPs) have also been shown to have other physiological functions, such as porin activity. OMPs have been shown to stimulate macrophages and lymphocytes to release a range of pro-inflammatory and immunomodulatory cytokines, including IL-1, IL-4, IL-6, CXCL8, TNF-α, GM-CSF and IFN-γ (9). OMPs can also promote resistance to complement-mediated killing by preventing activation of complement cascades and/or by blocking the formation of the membrane attack complex (49). A recent study reported that more than one-half of sera from patients with apical periodontitis lesions had strong reactions to *Porphyromonas gingivalis* cell components, especially RagB, which is a major outer membrane protein of this species (50). It was suggested that outer membrane proteins can be a possible virulence factor in apical periodontitis lesions.

Outer Membrane Vesicles

The release of membranous material from the outer surface of Gram-negative bacteria has been reported for a number of pathogenic species. This material has been referred to as vesicles or blebs. Vesicles are thought to be formed by extrusion of the outer membrane, arising from an imbalance between the growth of the outer membrane and other underlying cellular structures. In addition to containing LPS, outer membrane vesicles have a capacity to entrap contents of the periplasmic space, particularly lytic enzymes that break down large and impermeable molecules favoring their uptake as well as enzymes that confer resistance to antibiotics. Therefore, vesicles may afford bacteria a formidable virulence potential.

Lipoproteins

Lipoproteins are usually present in the cell wall of

Gram-negative bacteria and are responsible for anchoring the outer membrane to the peptidoglycan layer. Lipoproteins have been demonstrated to stimulate the release of IL-1β, IL-6, IL-12, and TNF-α by macrophages (9).

Fimbriae

Fimbriae are rod-shaped proteic structures originating in the cytoplasmic membrane and are composed of a single protein subunit termed fimbrillin. Distribution and numbers of fimbriae vary significantly, with some species showing 10 fimbriae per cell and others showing up to 1000 (51). Fimbriae are evenly distributed over the surface of the bacteria, but in some cases they are located preferentially on one part of the bacterial surface. Fimbriae are found mainly on Gram-negative bacteria, albeit they can also be present on certain streptococci and actinomycetes. The tip of the fimbriae mediates bacterial adherence to host tissue surfaces or other microorganisms, by attaching to specific receptors, usually in a lectin-like interaction. In addition to promoting adhesion, fimbriae have been demonstrated to elicit the release of cytokines by macrophages, including IL-1α, IL-1β, IL-6, CXCL8 and TNF-α (52).

Exopolysaccharides

Production of exopolysaccharides is a common characteristic of bacterial cells growing in their natural environment. Exopolysaccharides form highly hydrated, water insoluble gels. They may be formed by either homo- or heteropolysaccharides. Exopolysaccharides may have some important roles when it comes to bacterial pathogenicity. They may allow bacterial adhesion to host surfaces and may also serve as metabolic substrate in periods of starvation. In addition, exopolysaccharides (capsule) can play a crucial role in bacterial virulence by hindering phagocytosis or inhibiting complement activation and complement-mediated killing. Several bacterial species produce a capsule with a chemical structure that mimics host tissue, camouflaging the microorganism from the immune system. Exopolysaccharides can also stimulate cytokine synthesis by macrophages, including IL-1β, IL-6, CXCL8, and TNF-α (9), thus contributing to the damage to host tissues. Encapsulated bacteria have an increased ability to cause abscesses (53).

Flagella

Bacterial flagella are relatively long projections extending outward from the cytoplasmic membrane that confer motility to bacteria. The flagellum can rotate at speeds of up to 1200 rpm, thus enabling bacterial cells to move at speeds of 100 μm/s (54). In some bacteria, the flagella originate from the end of the cell – polar flagella, while peritrichous flagella are those that surround the cell. Although the basic structure of the bacterial flagellum appears to be similar for all bacteria, there are some structural variations that reflect bacterial diversity. For instance, the flagella of spirochetes do not extend from the cell but rather are inserted subterminally at each pole of the cell, wrap around the protoplasmic cylinder, and usually are long enough to overlap near the middle of the cell body. They are termed periplasmic flagella and are located between the protoplasmic cylinder and the outer sheath. Rotation of the periplasmic axial filament propels the cell by propagating a helical wave along the cell length so that it moves with a corkscrew motion. The periplasmic flagella of oral treponemes, in addition to being required for motility, have strong influences on cell helical morphology.

Main examples of oral bacteria that possess flagella and have motility include treponemes (periplasmic flagella), *Campylobacter rectus* and some other *Campylobacter* species (single polar flagellum), *Selenomonas* species (up to 16 lateral flagella forming a tuft), *Centipeda periodontii* (peritrichous flagella), some *Eubacterium* species (like *E. yurii*, which has a single polar flagellum), and species of the genera *Bacillus* and *Clostridium* (most having peritrichous flagella). Motility can be an important pathogenic trait of some species because it enables the bacteria to evade phagocytosis and invade the tissue. Furthermore, flagella may also induce the production of pro-inflammatory cytokines through a process involving recognition of flagellin by TLR5 (55).

Bacterial DNA

Bacterial DNA differs from mammalian DNA because of the presence of DNA motifs containing a central unmethylated CG dinucleotide (CpG). While CpG motifs are unmethylated and usually fairly abundant in bacterial DNA, they are methylated and highly suppressed in mammalian DNA. Moreover, the base

context of CpG nucleotides in the human genome is not random, with CpGs being most frequently preceded by a C or followed by a G, which is unfavorable for immune stimulation (56). Because of this, cells of the innate immune system can sense bacterial DNA and interpret its presence as infectious danger (57). Macrophages and dendritic cells can be directly stimulated by bacterial DNA to produce a variety of cytokines, such as IL-1β, TNF-α, IL-6, IL-1ra, IL-18, monocyte chemoattractant protein-1 and IFN-γ. Also, bacterial DNA has been demonstrated to be a potent B-cell mitogen (56). TLR-9 is involved in initiation of cellular activation by CpG DNA (58). Similar to LPS, CpG DNA modulates osteoclastogenesis in bone marrow cell/osteoblast co-cultures. Upon TLR-9 ligation, CpG DNA interacts with osteoblastic TLR-9 and elicits intracellular events leading to the increased expression of molecules regulating osteoclastogenesis (59).

Secreted Products

Enzymes

Several enzymes produced and released by bacteria may play a role in pathogenicity. *Proteinases* (or proteases) are enzymes either secreted extracellularly or expressed on the bacterial cell surface that are capable of hydrolyzing peptide bonds of proteins. They are candidates for contribution to the pathogenesis of apical periodontitis lesions through several mechanisms, including direct damage by degrading components of the extracellular matrix of the connective tissue; indirect damage by activating host matrix metalloproteinases; and subversion of the host defense mechanisms by inactivation of proteins such as immunoglobulins and complement components (60-62). For instance, bacteria with collagenase activity have been found more frequently in cases of large apical periodontitis lesions, which suggests that such enzyme activity may be contributory to the enlargement of the lesion (63). In addition to having direct and indirect harmful effects as well as protective activities against host defenses, bacterial proteinases can play a pivotal ecologic role in the habitat by acquiring nutrients in the form of peptides and amino acids that can be used by other species in the mixed bacterial consortium (64).

Several other enzymes can play a role in bacterial pathogenicity. *Hyaluronidase* is involved in the hydrolysis of hyaluronic acid, a constituent of the ground substance of the connective tissue. This enzyme can also be important for bacterial spread through tissues. Hashioka *et al.* (63) observed that bacteria with hyaluronidase activity were isolated from root canals with acute clinical symptoms. *Chondroitin sulfatase* and *acid phosphatase* are other enzymes that can be involved in degradation of components of the extracellular matrix of the connective tissue. *DNases* reduce the viscosity of debris from dead host cells (like in abscesses) and may thus allow the spread of bacteria within an area where extensive damage to host tissue has occurred. *Fibrinolysin* is produced by many hemolytic streptococci and activates a proteolytic enzyme of plasma, which is then able to dissolve coagulated plasma and probably is involved in the spread of the infection through tissues. *Phospholipases* can be associated with membrane damage of the host cells caused by cleavage of phospholipids, which destabilizes the membrane and results in cell lysis.

Exotoxins

Exotoxins are heat-labile polypeptides excreted by living bacteria and are highly antigenic and usually highly toxic. Leukotoxin is the most documented exotoxin known to play a role in the pathogenesis of periodontal diseases. Leukotoxin is cell-specific and binds to neutrophils, monocytes and a subset of lymphocytes, forming pores in the plasmatic membranes of these target cells. As a result of pore formation induced by the toxin, the cell loses the ability to sustain osmotic homeostasis and dies. Few oral bacteria, including *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Fusobacterium necrophorum*, and *Campylobacter rectus*, are recognized to produce exotoxins (67,68). Of these, only the latter has been frequently observed in endodontic infections (69).

Bacterial Peptide N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP)

fMLP peptides are derived from the N-terminal end of newly synthesized bacterial proteins. For some proteins, particularly those occurring in the cytoplasm, the fMLP peptides are cleaved posttranslationally and are not present in the mature protein. For membrane or secretory proteins, these N-terminal signal peptide ex-

tensions are cleaved by a peptidase following polypeptide transport across the membrane and, therefore, are released into the extracellular space. The signal peptide is incorporated in the protein in the form of a short-lived sequence extension and is believed to direct the transport of newly synthesized polypeptides across the membrane following their synthesis. fMLP is one such bacterial peptide and is mainly considered a strong chemoattractant and a powerful activator of polymorphonuclear leukocytes and macrophages (61,70,71).

Heat-Shock Proteins

Environmental stresses (*e.g.*, nutrient availability, temperature, pH, redox potential, etc.) may affect the survival of oral bacteria and induce the accumulation of damaged or denatured proteins. Prokaryotic and eukaryotic cells respond to these conditions by inducing or accelerating the synthesis of specific proteins known as stress proteins, including heat-shock proteins (HSPs). HSPs are families of highly conserved proteins whose main role is to allow microorganisms to survive under stress conditions. They act as molecular chaperones in the assembly and folding of proteins, and as proteases when damaged or toxic proteins have to be degraded. Many HSPs are expressed constitutively under normal conditions but are rapidly up-regulated under stressful conditions.

HSPs are not exclusively intracellular proteins, but may also be located on the cell surface and on outer membrane vesicles released by bacteria (72). They are commonly grouped into families based on molecular weight: small HSPs, GroES-homologue proteins or HSP10 (~10 kDa), DnaJ-homologue proteins or HSP40 (~40 kDa), GroEL-homologue proteins or HSP60 (~60 kDa), DnaK-homologue proteins or HSP70 (~70 kDa), HptG-homologue proteins or HSP90 (~90 kDa), and Clp ATP-dependent proteases (HSP100) (72). Many HSPs of candidate endodontic pathogens, including *Tannerella forsythia, Campylobacter rectus, Candida albicans, Prevotella intermedia, Prevotella nigrescens, Prevotella buccae, Porphyromonas gingivalis, Fusobacterium nucleatum* and *Treponema denticola*, have been identified.

HSPs may play different roles as a virulence factor. A number of bacteria appear to use some specific HSPs as adhesins (73). In addition to participating in bacterial adhesion, HSPs have been shown to have cell-cell signaling properties and are able to modulate the activity of host cells. The actions of bacterial HSPs on host cells include inducing the synthesis of pro-inflammatory cytokines (74,75). HSPs have also been reported to promote apoptosis and this effect is likely to inhibit host antibacterial responses (73). The cytotoxicity of some bacterial HSPs may also contribute to tissue destruction, whereas the presence of common epitopes in host proteins and microbial HSPs may lead to pathological autoimmune responses (72).

Metabolic End-Products

Several end-products of bacterial metabolism are released to the extracellular environment and may be toxic to host cells, cause degradation of constituents of the extracellular matrix of the connective tissue, and interfere with the host defense processes (76-78). Among diverse bacterial end-products, volatile sulfur compounds, short-chain fatty acids, polyamines, indole and ammonia have been regarded as putative virulence factors.

Volatile sulfur compounds are formed as a result of desulfuration of amino acids containing sulfhydryl groups. For instance, hydrogen sulfide is derived from desulfuration of cysteine and methyl mercaptan from desulfuration of methionine. It has been demonstrated that innumerous oral bacterial species, including candidate endodontic pathogens, are able to form volatile sulfur compounds (79,80). Examples include: *Treponema denticola, Tannerella forsythia, Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum*, *Parvimonas micra* (formerly *Peptostreptococcus/Micromonas micros*), *Actinomyces* species, and *Eubacterium* species (79). Volatile sulfur compounds are expected to be formed by endodontic bacteria from the free sulfur amino acids present in tissue fluids or exudate that penetrate in the root canal. Proteolytic activity of some bacterial species in the root canal can also supply other bacteria with suitable substrates for production of volatile sulfur compounds. These substances can be highly toxic to host cells (81). Although no study seems to exist as to the production of volatile sulfur compounds in infected root canals, it is entirely possible that these compounds may accumulate in necrotic root canals and reach toxic levels to the periradicular tissues.

Short-chain fatty acids [CH_3-$(CH_2)_x$-COOH, x<3 thus C≤5] released by endodontic bacteria into the environment include volatile acids (propionic, butyric, isobutyric, valeric, isovaleric, acetic, and formic acids) and non-volatile acids (lactic and succinic acids) (82). Short-chain fatty acids may have a broad range of biological effects. Butyric, propionic and valeric acids have been demonstrated to exhibit cytotoxic effects on Vero cells (76). Short-chain fatty acids, and butyric acid in particular, can cause inhibition of T-cell proliferation and induce the production of pro-inflammatory cytokines by monocytes (78). Butyric acid may also induce apoptosis in splenic T- and B-cells (83-85). Succinic acid at concentrations commonly found in clinical abscesses has been shown to impair the antimicrobial activity of neutrophils (86,87).

Polyamines are produced by microorganisms as a result of amino acid decarboxylation by decarboxylases. Examples of polyamines include putrescine, spermidine, spermine and cadaverine. Putrescine [NH_2 $(CH_2)_4$ NH_2] is synthesized directly from ornithine by ornithine decarboxylase and indirectly from arginine by arginine decarboxylase and 2 aminopropyltransferases. Spermidine [NH_2 $(CH_2)_3$ NH $(CH_2)_4$ NH_2] and spermine [NH_2 $(CH_2)_3$ NH $(CH_2)_4$ NH $(CH_2)_3$ NH_2] are formed by the subsequent addition of an aminopropyl moiety to putrescine and spermidine, respectively. The diamine cadaverine [$NH_2(CH_2)_5NH_2$] can be formed from lysine either via the action of ornithine decarboxylase or as a result of lysin and decarboxylase activity. These enzymes are present in several bacteria, including species of the genera *Enterococcus, Pseudomonas*, *Lactobacillus*, *Escherichia*, and *Staphylococcus*. Polyamines may be involved in the maintenance of cell viability, stimulation of cell proliferation, and modulation of inflammatory processes (88,89). Polyamines can dysregulate apoptosis of polymorphonuclear leukocytes and lead to premature cell death (90). A study has identified and quantified polyamines in infected root canals and demonstrated that greater amounts were detected in teeth with spontaneous pain, swelling and foul odor when compared to asymptomatic teeth (88). The authors suggested that intracanal polyamines, especially putrescine, might leak out through the apical foramen and be involved in the development of pain.

Other end-products released by bacteria, such as indole (C_8H_7N) and ammonia (NH_3), can also be toxic to host cells (77,91).

The composition of the bacterial consortium within infected root canals will certainly dictate the type of end-products present as well as their concentration. On the one hand, some compounds may be consumed by other species and be further degraded. On the other hand, metabolites can be left to accumulate and reach toxic levels to the periradicular tissues. Accumulation of bacterial metabolic end-products in the infected root canal may thus represent an additional pathogenic mechanism of the root canal microbiota. Many of these end-products, such as short-chain fatty acids, polyamines and particularly volatile sulfur compounds, are responsible for the foul smell typical of endodontic anaerobic infections.

CONCLUDING REMARKS

Apical periodontitis is a multifactorial disease that is resultant of the interplay of many host and bacterial factors. Although LPS is undoubtedly the most studied and quoted virulence factor, it sounds simplistic to ascribe to this molecule all responsibility for apical periodontitis causation. This statement is further reinforced by the fact that some cases of primary infections and many cases of secondary/persistent infections harbor exclusively Gram-positive bacteria. Therefore, the involvement of other factors must not be overlooked. In fact, the pathogenesis of different types of apical periodontitis and even the same type in different individuals is unlikely to follow a stereotyped fashion with regard to the involved bacterial mediators. Here were presented several factors that can act together to cause the diverse stages of the disease process, with consequent signs and symptoms. No single species possesses or expresses all the factors mentioned here and Table 3 lists the main virulence factors of some important candidate endodontic pathogens. Thus, which factors are involved in each specific case will depend on the composition of the microbiota, *i.e.*, the bacterial species present in the necrotic root canal. It is still worth pointing out that the potency of biological effects of the same virulence factor (*e.g.*, LPS) can significantly vary from species to species (92,93). Thus, given the nonspecific nature of primary endodontic infections, different combinations of virulence factors will cause disease in different subjects. Knowledge of the main factors involved in the pathogenesis of apical periodontitis may help establish proper therapeutic measures to inactivate this bacterial "artillery".

Table 3. Main virulence factors of some important endodontic pathogens.

Microorganism	Type of endodontic infection	Putative virulence factor
Treponema denticola	Primary	major surface protein; chymotrypsin-like protease complex; extracellular or membrane-associated proteolytic and hydrolytic enzymes; lipooligosaccharide; lipoprotein; phospholipases; metabolites (acetic and lactic acids, H_2S); flagella; heat-shock proteins
Tannerella forsythia	Primary	lipopolysaccharide; trypsin-like enzyme; acid phosphatase; metabolites (acetic, propionic, butyric, isovaleric and phenylacetic acids); apoptosis-inducing factor; heat-shock proteins
Porphyromonas endodontalis	Primary	lipopolysaccharide; capsule; outer membrane proteins; proteinases; outer membrane vesicles; acid phosphatase; metabolites (butyric and propionic acids, indole, H_2S)
Porphyromonas gingivalis	Primary	lipopolysaccharide; fimbriae; capsule; lipoproteins; outer membrane vesicles; proteinases; fibrinolysin; phospholipase; acid phosphatase; dnase; hyaluronidase; chondroitin sulfatase; hemolysins; metabolites (H_2S, methylmercaptan, dimethyl disulfide, butyric and propionic acids, indole, ammonia); heat-shock proteins
Parvimonas micra	Primary	peptidases; hyaluronidase; capsule; H_2S
Prevotella intermedia/ nigrescens	Primary	lipopolysaccharide; fimbriae; metabolites (indole, H_2S, ammonia, acetic and succinic acids); proteinases; hemolysins; acid phosphatase; phospholipase; heat-shock proteins
Fusobacterium nucleatum	Primary	lipopolysaccharide; outer membrane proteins; capsule; metabolites (butyric and propionic acids, ammonia, indole); heat-shock proteins
Campylobacter rectus	Primary	extracellular cytotoxin; lipopolysaccharide; S-layer; arylsulfatase; H_2S; heat-shock proteins
Streptococcus anginosus group	Primary/Persistent/Secondary	peptidoglycan; lipoteichoic acid; enzymes; metabolites
Enterococcus faecalis	Persistent/Secondary	lipoteichoic acid; gelatinase; hyaluronidase; cytolysin; aggregation substance; pheromones; heat-shock proteins
Candida albicans (yeast)	Persistent/Secondary	mannose-containing proteins; mannan; phospholipase; proteinases; hyaluronidase; acid phosphatase; chondroitin sulfatase; phospholipase; heat-shock proteins

RESUMO

Lesões perirradiculares compreendem um grupo de doenças inflamatórias causadas por microrganismos (principalmente bactérias) infectando o sistema de canais radiculares com polpa necrosada. É improvável que a patogênese dos diferentes tipos de lesão perirradicular, e até mesmo daquelas do mesmo tipo, mas em diferentes indivíduos, obedeça um padrão estereotipado com relação aos mediadores bacterianos envolvidos. A patogênese destas doenças é complexa e envolve inúmeros fatores relacionados às bactérias e ao hospedeiro. Este artigo de revisão discute a patogênese bacteriana das lesões perirradiculares agudas e crônicas, enfatizando os fatores bacterianos que estão possivelmente envolvidos nos diferentes estágios do processo infeccioso, incluindo produtos secretados (enzimas, exotoxinas, peptídeos N-formilados, proteínas de choque térmico e produtos terminais do metabolismo) e componentes estruturais (lipopolissacarídeo, peptidoglicano, ácido lipoteicóico, lipoproteínas, fímbrias, flagelos, proteínas e vesículas de membrana externa, DNA e exopolissacarídeos). O conhecimento dos fatores bacterianos envolvidos na patogênese das lesões perirradiculares é importante para o entendimento do processo patológico bem como para ajudar no estabelecimento de medidas terapêuticas adequadas para desativação desta "artilharia" bacteriana.

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