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ORIGINAL ARTICLE

Oral Microbial Interactions

The Oral Microbial Consortium's Interaction with the Periodontal Innate Defense System

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The oral microbial consortium is the most characterized polymicrobial microbial community associated with the human host. Extensive sampling of both microbial and tissue samples has demonstrated that there is a strong association between the type of microbial community found in the gingival crevice and the status of innate host mediator expression. The strong clinical association between the microbial community and the innate host response in both clinically healthy and diseased tissue suggests that the oral consortium has a direct effect on periodontal tissue expression of innate defense mediators. A preliminary study in germ-free mice has demonstrated that the oral commensal consortium has direct effect on IL-1β expression, indicating that this microbial community may contribute to the strong protective status of healthy gingival tissue. Likewise, the lipopolysaccharide composition and invasion characteristics of *Porphyromonas gingivalis*, an oral bacterium strongly associated with periodontitis, suggest that it may be a keystone member of the oral microbial community and facilitate a destructive change in the protective gingival innate host status.

The Microbial Oral Community Is the Most Completely Characterized Group of Bacteria That Persistently Colonize the Host

Examining the potential symbiotic relationships in the oral cavity is greatly aided by the extensive studies that have characterized the composition of dental plaque. Dental plaque is an oral microbiological consortium that forms a biofilm on the tooth and tooth root surface. The first characterization of dental plaque was performed by van Leeuwenhoek in 1683 where he described gingival bacteria as "animacules" that contributed to the beginning of the science of bacteriology (Dobell, 1958). Subsequently, descriptive studies performed throughout the twentieth century demonstrated that dental plaque was a distinct structure containing layers of different morphological types that formed on the tooth and tooth root surface in an orderly ecological succession (Socransky and Haffajee, 1994). Microbiological analyses revealed that the composition of commensal oral bacteria and the bacterial load isolated from healthy sites is significantly different from that found in diseased sites. In healthy sites the microbial load is low (10^2–10^3 isolates may be cultured from an individual healthy sulcus) (Darveau *et al*., 1997) consisting of mostly gram-positive streptococci (e.g., *Streptococcus gordonii*) and Actinomyces with about 15% gram-negative rod species, including *Fusobacterium nucleatum*. In contrast, characterization of the periopathogenic microbial flora has revealed that the microbial load is higher (10^5–10^8 microorganisms may be cultured from an individual pocket), and there is an increase in the number of gram-negative organisms (15–50%) (Tanner *et al*., 1996; Darveau *et al*., 1997) when compared to clinically healthy sites. Further, the relative ease of sampling from the oral cavity combined with DNA probe analysis of bacterial populations facilitated multiple cluster and community ordination statistical methods (Socransky *et al*., 1998) and defined the previously characterized shift from mostly gram-positive to gram-negative species (Socransky and Haffajee, 1994) that occurs in the transition from periodontal health to disease. These analyses identified periopathogenic bacteria, including *Tannerella forsythia*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Prevotella intermedia* (Socransky *et al*., 1998), that group together in diseased sites (Ximenez-Fyvie *et al*., 2000). These studies also laid a foundation for studies that utilize molecular techniques to identify noncultivatable oral bacteria (Kroes *et al*., 1999), define oral transmission routes (Li and Caufield, 1998), and examine associations between species genotypes and disease (Griffen *et al*., 1999). In addition, advances in our understanding of biofilm structure have led to studies examining regulation of specific microbial adhesions

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(McNab *et al*., 2003) and how host microenvironmental conditions influence the microarchitecture of the dental plaque biofilm (Blehert *et al*., 2003). These studies validate that the microbial community associated with oral clinical health is not a random assortment of bacteria but rather represents a highly organized microbial consortium that has evolved to live with each other to occupy niches in the oral host environment.

Innate Host Defense Status in Clinically Healthy and Diseased Tissue

Similar to the highly organized microbial consortium associated with clinically healthy tissue, the innate defense status of this tissue is also highly organized and regulated (Fig. 1). The relative ease of tissue sampling from the oral cavity and the identification of innate host response and inflammatory molecular mediators allowed a comprehensive characterization of the tissue status in both gingival health and disease. Clinically healthy periodontal tissue contains a unique expression of select inflammatory mediators. Early histological studies (Page and Schroeder, 1976) of clinically healthy tissue demonstrated that it contains a cellular infiltrate located in juxtaposition to the colonized tooth surface (Kornman *et al*., 1997b). A portion of this cellular infiltrate has been described as forming a wall of neutrophils precisely located between bacteria and residing just outside the junctional epithelium, the epithelial cell surface closest to the dental plaque biofilm (Kornman *et al*., 1997a). Consistent with these observations molecular characterization of healthy periodontal tissue has demonstrated that IL-8, ICAM, and E-selectin are expressed in clinically healthy tissue (Moughal *et al*., 1992; Nylander *et al*., 1993; Gemmell *et al*., 1994; Tonetti *et al*., 1994; Tonetti, 1997). These inflammatory mediators are necessary for leukocyte diapedesis from the vasculature and directed movement through tissue. E-selectin expression on endothelial cells facilitates a tethering interaction between the leukocyte and the endothelial cell wall initiating the rolling stage required for leukocyte exit (Springer, 1994). IL-8 is a key neutrophil chemoattractant, and ICAM facilitates cellular adhesion. It has been demonstrated that a gradient of IL-8 and ICAM-1 expression exists in clinically healthy tissue (Tonetti *et al*., 1998). IL-8 expression was greatest at the most superficial junctional epithelial cell layers, and the levels of ICAM-1 increased toward areas exposed to bacterial challenges. More additional immunohistochemical and *in situ* studies have revealed that clinically healthy periodontal tissue also expresses human β defensin molecules 1, 2, and 3 (Lu *et al*., 2004, 2005) as well as soluble (Jin and Darveau, 2001) and membrane bound CD14 (Jin *et al*., 2004) and lipopolysaccharide binding protein (Ren *et al*., 2004). Lipopolysaccharide binding protein expression was greatest in the gingival epithelium (Ren *et al*., 2004). These innate defense proteins function in either bacterial killing or bacterial removal, consistent with the notion that healthy periodontal tissue is armed by the innate host defense system to protect against bacterial infection. Clinically healthy human gingival tissue also expresses low levels of TLR2 (Ren *et al*., 2005; Sugawara *et al*., 2006): while expression of TLR4 was reported in one study (Sugawara *et al*., 2006), the other (Ren *et al*., 2005) did not observe expression of this innate host defense receptor in healthy tissue. A more recent study (Beklen *et al*., 2008) describes the expression of TLR's 1–10 in both clinically healthy and diseased tissue. In addition, NOD1 and NOD2 act synergistically with select TLR's resulting in the expression of antimicrobial peptides in response to microbial challenge (Uehara and Takada, 2008). Further, gingival fibroblasts are well equipped to respond to bacterial components and may contribute to the IL-8 observed in clinically healthy tissue (Mahanonda *et al*., 2007). These data are all consistent with the notion that innate host defense mediator expression in clinically healthy tissue is key to the maintenance of periodontal health.

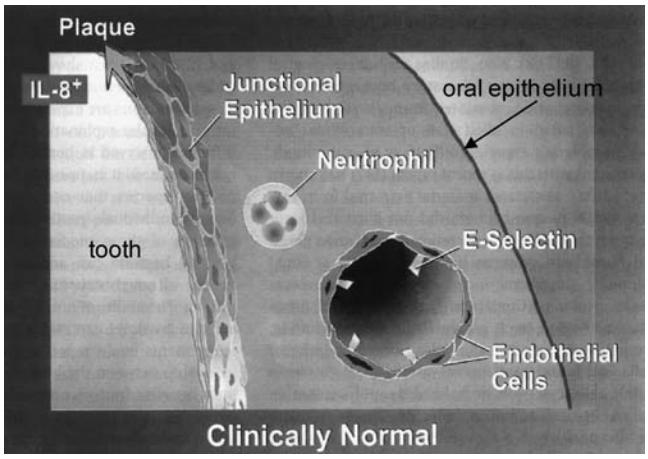

FIG. 1. Innate host defense status in clinically normal tissue. Recently, it has been demonstrated that clinically healthy tissue displays low level expression of select inflammatory mediators. The expression of E selectin on the vascular endothelium, for example, is believed to facilitate leukocyte exit from the vasculature into surrounding tissue where they remove bacteria. A gradient of IL-8 expression (indicated by shades of gray) exists in normal tissue to guide leukocytes to the site of bacterial colonization. Recent evidence (Darveau *et al.*, unpublished) suggests that the biofilm of gingival health may provide the stimulus for expression of these mediators, suggesting a commensal relationship between the host and these bacteria. This figure is based on the work of Tonetti *et al.* (1994) and Moughal *et al.* (1992). Reprinted with permission from Darveau *et al*. (1997).

The contribution of the innate defense status observed in clinically healthy tissue to periodontal health is highly significant. Loss of the protective neutrophilic barrier function either by congenital deficiency (Page *et al*., 1987; Waldrop *et al*., 1987; Carrassi *et al*., 1989; Hart *et al*., 1994) or by chemical induction with antimitotic agents such as cyclophosamide (Attström and Schroeder, 1979; Sallay *et al*., 1984; Hemmerle and Frank, 1991; Yoshinari *et al*., 1994) invariably leads to disease. Further, studies have shown that the lack of an intact innate host defense system may be responsible for the significantly increased incidence of severe periodontitis observed in diabetic patients (type I and type II) and tobacco users (Bergstrom *et al*., 1988; MacFarlane *et al*., 1992; Offenbacher *et al*., 1996; Zambon, 1996; Salvi *et al*., 1997).

Similar to the association found between the commensal oral microbial flora and the innate host defense status of clinically healthy tissue, there is a strong correlation between the periopathogenic microbial flora and a destructive inflammatory response (Ximenez-Fyvie *et al*., 2000). The innate defense status found in tissue obtained from periodontitis

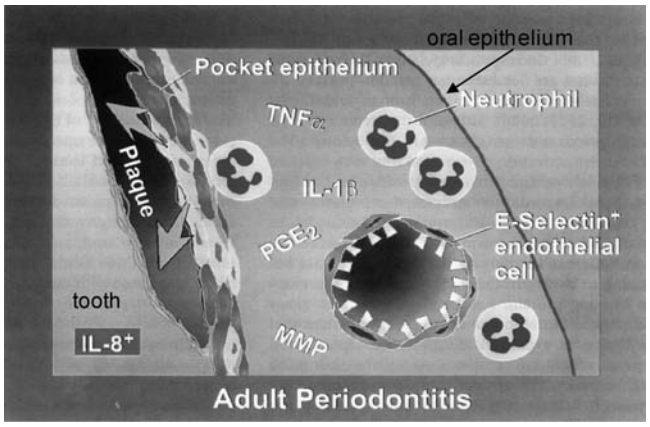

FIG. 2. Innate host defense status in adult periodontitis. In adult periodontitis, the molecular mediators of inflammation that are expressed in clinically healthy tissue are expressed at higher levels, and new mediators are present. The gradient of IL-8 expression found in healthy tissue is disrupted (see Fig. 1 legend), and a pocket epithelium forms. This figure is based on the work of Tonetti *et al.* (1994) and Moughal *et al.* (1992). TNFα, tumor necrosis factor α; PGE2, prostaglandin E2; MMP, matrix metalloproteinase. Reprinted with permission from Darveau *et al*. (1997).

sites is significantly different from that found in healthy tissue (Fig. 2). Periodontitis is associated with the expression of more and different inflammatory mediators compared to clinically healthy tissue. This has contributed to the notion that periodontal disease is a result of both bacterial and host response factors (Page *et al*., 1997). Although there is increased expression of IL-8 and ICAM, the characteristic expression pattern of these mediators observed in clinically healthy tissue is absent in tissue obtained from periodontitis sites (Tonetti *et al*., 1994, 1998). There is no IL-8 gradient, but rather patches of intense IL-8 expression are observed in the gingival epithelium with areas of no or little expression (Tonetti *et al*., 1994). ICAM and E selectin are both expressed at much higher levels than that observed in healthy tissue (Tonetti *et al*., 1994). In addition, there is an increase in the expression levels of both TLR2 and TLR4 in periodontitis sites compared to clinically healthy tissue (Ren *et al*., 2005; Sugawara *et al*., 2006). The increase in these innate host receptors may have profound effects on the innate host response of periodontal tissue. Evidence that these mediators contribute to the characteristic loss of connective tissue and the alveolar bone that surrounds and supports the tooth root associated with periodontal disease comes from studies that demonstrate inflammatory mediator levels decrease after successful treatment (Offenbacher *et al*., 1986; Masada *et al*., 1990). Further, administration of antiinflammatory drugs that reduce levels of these mediators can suppress bone and tissue destruction (Offenbacher *et al*., 1987), and nonsteroidal antiinflammatory drugs that block prostaglandin synthesis can arrest tissue destruction (Offenbacher *et al*., 1987). Finally, removal of dental plaque remains the most effective mechanism of restoring an appropriate innate host response in periodontitis patients (Page *et al*., 1997), providing more evidence that the bacterial composition associated with periodontitis is directly responsible for a dysfunctional innate host response.

Therefore, there are strong correlations between the type of microbial consortium found in the gingival pocket and the status of innate mediator expression in the adjacent periodontal tissue. Since the gingival epithelium does not contain tight junctions, bacterial components shed from the dental plaque biofilm can penetrate gingival tissue (Schwartz *et al*., 1972; Moore *et al*., 1986; Wilson *et al*., 1986; McCoy *et al*., 1987; Hamada *et al*., 1990), providing a mechanism by which this tissue may sample the oral bacterial plaque composition. It seems likely, therefore, that the oral microbial consortiums found in clinically healthy and diseased tissues contribute to both the highly orchestrated innate mediator expression found in healthy sites and the destructive innate mediator expression found in diseased tissues.

The Use Germ-Free Mice to Define Direct Consortium Effects on Gingival Tissue

The most definitive approach to determine the contribution of the oral microbial consortiums to the innate host defense status in the periodontium is the use of germ-free mice. For example, germ-free mice have been employed to determine the contribution of commensal bacteria to normal intestinal innate defense, and immune development has been carefully studied with the use of germ-free mice (Gordon and Pesti, 1971; Umesaki and Setoyama, 2000; Hooper *et al*., 2001; Xu and Gordon, 2003; Macpherson and Harris, 2004). Germ-free mice that are completely devoid of bacteria are generated by sterile Caesarean section and raised aseptically in an isolator with sterile filtered air and are housed using sterile food, water, and bedding. Germ-free mice are distinct from specific-pathogen-free mice that are only devoid of known mouse pathogens and contain intestinal bacteria (Macpherson and Harris, 2004).

It has been shown that commensal bacteria are required for the complete development of Peyer's patches, the lamina propria, and the intraepithelial spaces, all three of the main immune elements found in the intestine (Duncan and Edberg, 1995; Falk *et al*., 1998). Studies in germ-free mice have revealed that the commensal bacteria induce angiogenesis, contributing to the development of the complex vascular beds found just underneath the mucosal surface (Stappenbeck *et al*., 2002). Further, it has been found that constitutive ICAM-1 expression in these vessels is also regulated by the presence of the commensal microbiota (Komatsu *et al*., 2000). In fact, the state of controlled inflammation that normally exists in the intestine has been attributed to both the quality and quantity of intestinal commensal microorganisms (Chadwick and Anderson, 1992; Cebra, 1999). These studies demonstrate that commensal colonization of the intestine orchestrates selective expression of innate host defense components facilitating a mature tissue state that provides immune protection for the host.

However, little is known concerning the contribution of oral commensal bacteria to the armed protective state observed in healthy human periodontal tissue. Germ-free mice have been extensively utilized by oral researchers, however, in the context of caries and periodontitis disease models (Niederman *et al*., 2001) as opposed to elucidation of direct effects of bacterial colonization on innate host mediator expression in periodontal tissue. Nevertheless, these early disease models in germ-free mice combined with periodontitis mouse models of infection provide evidence that mouse

commensal bacteria influence periodontal tissue innate host mediator expression. For example, the *P. gingivalis* gavage model developed by Baker (Hart *et al*., 2004) has shown that prior antibiotic treatment to reduce the commensal load is necessary to facilitate *P. gingivalis* colonization of the oral cavity, mRNA expression of many innate defense mediators is elevated in healthy gingival mouse tissue (Hart *et al*., 2004), and there are differences in the susceptibility to periodontal infection that are genetically inherited among different inbred mouse strains (Baker *et al*., 2000). However, these studies cannot differentiate between the passive colonization resistance effect of commensal colonization and the direct development of the protective or destructive innate defense status found in periodontal tissue by oral commensal bacteria.

We have conducted a preliminary investigation directly comparing innate host mediator expression in germ-free and conventionally reared mice (Dixon *et al*., 2004). It was found that IL-1β levels were significantly higher in conventionally reared mice, consistent with the notion that commensal bacteria in the periodontium, similar to the intestine, actively participate in establishing the innate mediator expression of clinically healthy or normal periodontal tissue. The reasons only IL-1β was identified as being differentially expressed in germ-free and conventionally reared mice included a limited number of samples examined in the pilot study. The presence of elevated amounts of this inflammatory mediator in healthy conventionally reared animals compared to germ-free controls may appear paradoxical since it has been associated with the development of periodontitis (Masada *et al*., 1990). However, we suspect that the presence of IL-1β in clinically healthy periodontal tissue may serve as a priming mechanism for several different cell types found in the periodontium. These data suggest that the host genetically programs select cytokine expression in the absence of bacterial colonization that then may be altered depending upon the number and type of bacterial species colonizing host tissue.

P. gingivalis Responds to Hemin by Generating a TLR4 Antagonistic Lipid A Structure

We have been examining the potential contribution of *P. gingivalis*, an oral bacterium strongly associated with periodontitis, to altering the innate defense status of periodontal tissue. A likely candidate for modulating innate mediator expression in host tissue is lipopolysaccharide (LPS). Indeed, LPS has been termed a pattern recognition receptor ligand for the innate host defense system (Medzhitov and Janeway, 2000). The concept of pattern recognition, originally put forward by Janeway (Janeway, 1992), proposes that the host has evolved receptors that recognize common conserved structures found in a variety of different microbes. LPS is present in all gram-negative bacteria, is essential for bacterial viability (one with notable exception [Steeghs *et al*., 1998]), and contains a highly conserved lipid A structure consisting of a phosphorylated beta-(1,6)-glucosamine disaccharide substituted with hydroxylated and nonhydroxylated fatty acids (Takada and Kotani, 1992), completely filling the criteria for innate host recognition. Consistent with its proposed role as a sentry employed by the host to monitor bacteria infection, *in vitro* studies have confirmed that whole bacteria and their respective isolated LPSs yield similar responses (Darveau *et al*., 1991; Somerville *et al*., 1996), and *in vivo* studies have validated the important role LPS serves in host recognition of bacterial infection (Khan *et al*., 1998; Somerville *et al*., 1999; Haziot *et al*., 2001). Clearly, innate host recognition of LPS is a key initiating event for the subsequent clearance of gram-negative bacteria from infected host tissues.

We have found that *P. gingivalis* changes its lipid A structural composition in response to the hemin concentration in the medium. Hemin binds host iron and represents the major iron acquisition system for *P. gingivalis* (Olczak *et al*., 2005). Hemin is a relevant microenvironmental factor for *P. gingivalis* since its concentration is low in healthy tissue and high in diseased sites where vascular ulceration leads the leakage of blood into the underlying gingival epithelium. The lipid A structural content of *P. gingivalis* after growth in medium containing 1 μg/mL hemin consists of a single major monophosphoryl penta-acylated lipid A cluster (centered at m/z 1690), while the lipid A content of bacteria incubated with 10 μg/mL hemin showed a significantly reduced amount of this lipid A structure and a significant increase in both monophosphoryl tetra-acylated lipid A structures (m/z 1435 and 1449) and a diphosphoryl penta-acylated lipid A cluster (centered at m/z 1770). The monophosphoryl penta- and tetra-acylated lipid A structures have been purified and shown to display distinctly different effects on endothelial cell E selectin activation (Reife *et al*., 2006) due to the fact that PgLPS1435/1449 is a TLR4 antagonist (Darveau *et al*., 1995; Yoshimura *et al*., 2002; Coats *et al*., 2003; Reife *et al*., 2006), whereas PgLPS1690 is a TLR4 agonist (Reife *et al*., 2006). Consistent with PgLPS1435/1449 and PgLPS1690 displaying different effects on E-selectin expression, it was shown that these two LPS preparations interact with the TLR4 complex differently (Reife *et al*., 2006). The expression of functionally distinct lipid A structures that have opposing effects on TLR4 activation indicates that *P. gingivalis* may utilize its lipid A structural content to modulate the innate host response in different microenvironmental conditions.

P. gingivalis May Represent a Keystone Species in the Oral Microbial Consortium

Two lines of evidence indicate that *P. gingivalis* may be a keystone species in the oral microbial community. Keystone species in this context means that this one bacterium serves an essential function for the entire community, similar to a differentiated cell serving a function for an entire tissue. The first is that the presence of the *P. gingivalis* TLR4 lipid A antagonist that can block TLR4 activation in response to several different oral microbial bacteria (Darveau *et al*., 1995) through competitive binding to MD-2 (Coats *et al*., 2005, 2007) combined with the observation that *P. gingivalis* releases LPS that can penetrate gingival tissue (Schwartz *et al*., 1972) supports the notion that the TLR4 lipid A antagonist will dampen TLR4 responses for the entire oral microbial community. This is especially relevant considering the proposal by Munford (Munford and Varley, 2006) that TLR4 sensing prevents invasion into submucosal tissue by mucosal gram-negative bacteria. Therefore, hemin may act as an environmental sensor that *P. gingivalis* responds to by making the lipid A TLR4 antagonist, and then this facilitates invasion of tissue and modulation of innate host defense mediator expression in response to numerous members of the oral microbial consortium. Hemin, provided to the bac-

teria in the form of hemoglobin, has been shown to significantly increase in concentration in diseased sites (Hanioka *et al*., 1990, 1991). Secondly, we have shown that when *P. gingivalis* invades gingival epithelial cells it blocks the epithelial cell IL-8 response to other oral bacteria (Darveau *et al*., 1998). We have termed this process local chemokine paralysis, since the ability to detect and locate bacterial colonization by IL-8 would be effectively paralyzed and unable to function at sites of *P. gingivalis* invasion. Inhibition of IL-8 accumulation by *P. gingivalis* at sites of bacterial epithelial cell invasion could have a devastating effect on innate host defense in the periodontium, where bacterial exposure is constant. The host may no longer be able to detect the presence of bacteria and direct leukocytes for their removal. This phenomenon represents another mechanism by which the action of a single bacterial member of the oral consortium can affect the host responses to a wide variety of different bacteria.

Disclosure Statement

No competing financial interests exist.

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