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Review Article

Multispecies biofilms and host responses: "Discriminating the Trees from the Forest"

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ABSTRACT

Periodontal diseases reflect a tissue destructive process of the hard and soft tissues of the periodontium that are initiated by the accumulation of multispecies bacterial biofilms in the subgingival sulcus. This accumulation, in both quantity and quality of bacteria, results in a chronic immunoinflammatory response of the host to control this noxious challenge, leading to collateral damage of the tissues. As knowledge of the characteristics of the host-bacterial interactions in the oral cavity has expanded, new knowledge has become available on the complexity of the microbial challenge and the repertoire of host responses to this challenge. Recent results from the Human Microbiome Project continue to extend the array of taxa, genera, and species of bacteria that inhabit the multiple niches in the oral cavity; however, there is rather sparse information regarding variations in how host cells discriminate commensal from pathogenic species, as well as how the host response is affected by the three-dimensional architecture and interbacterial interactions that occur in the oral biofilms. This review provides some insights into these processes by including existing literature on the biology of nonoral bacterial biofilms, and the more recent literature just beginning to document how the oral cavity responds to multispecies biofilms.

1. Introduction

Periodontal diseases are one of the most frequent global infections in humans [1,2]. While many medically important infections occur on a global scale, most of these are due to monoinfections with specific microbial pathogens, e.g. *Vibrio cholerae*, *Mycobacterium tuberculosis*, influenza. Moreover, few of these are chronic infections of the host, although there are notable pathogens that represent long term disease processes, e.g. *Plasmodium falciparum*, HIV. An even more select subset of pathogens initiate a disease process by creating organized biofilms that enhance their ability to adhere, replicate, accumulate, and express their virulence potential [3–6]. However, even in this scenario, most medically significant pathogens that form biofilms tend to be monospecies infections, e.g. *Pseudomonas aeruginosa, Staphylococcus aureus, Yersinia pestis*.

Chronic periodontal infections provide a very different "face" to the host with regards to controlling these bacteria. First, it is estimated that over 700 species of bacteria can colonize the oral cavity of humans, and that in an individual the range of niches in the oral cavity host 100 or more species [7]. Second, these bacteria accrete and accumulate on host surfaces with tropisms for specific sites in the oral cavity, as well as specificity for interbacterial interactions that result in three-dimensional structured and organized biofilms [8–10]. Third, the bacteria must survive within a milieu of host factors derived from saliva, serum, and gingival tissues [11]. Finally, this dynamic host-bacterial environment has evolved to help sustain a protective commensal microbial ecology, while minimizing the ability of opportunistic pathogens to emerge within these established biofilms [12]. The majority of data derived from studies of host-bacterial interactions in periodontitis has focused on the individual "trees" in isolation and have provided extensive information in controlled systems to delineate the capacity of single species to alter host cell functions. However, not available in these investigations was the ability to define how the microbial "forest" as a multicellular complex three-dimensional structure could present a challenge quite different from simply the sum of the species in the forest. In order to extend our understanding of these interactions that are required to maintain homeostasis in the oral cavity, and the characteristics of a dysregulated response that leads to disease progression, it is necessary to begin to model the host cell-biofilm attributes at a molecular level.

2. Medically important pathogenic biofilms

Acute medical infections are generally associated with planktonic bacteria and must be diagnosed rapidly and treated accurately to prevent tissue damage and/or death. In contrast,

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bacteria in biofilms demonstrate an infectious course usually accompanied by sustained host hyper-inflammation, with the biofilm species using plasma exudate as nutrition. This lifestyle is a particularly important bacterial adaptation to grow as part of a sessile community, which mimics an integrated multicellular organism with its own development cycle, cooperative behavior among the species, and coordinated management using quorum sensing signal molecules to communicate among the constituents. These microorganisms attach to both biotic and abiotic surfaces, followed by multiplication while becoming embedded in an extracellular matrix, leading to characteristic biofilms. The outcomes are three-dimensional aggregates of bacteria that are now recognized as significant contributors to in many bacterial-associated infections (e.g. endocarditis, dental caries, middle ear infections, osteomyelitis, medical device-related infections, chronic lung infections in cystic fibrosis patients, persistence of food-borne pathogens) [13–16]. Recent estimates suggest that 60–85% of all microbial infections involve biofilms developed on these natural tissues or artificial devices.

Historical studies of biofilms based upon medical infectious agents were described as sessile communities of microbes characterized by cells that are irreversibly attached to a substratum or coaggregate with each other. Generally they are embedded in a matrix of extracellular polymeric substances. Additionally, the initial bacterial adherence and biofilm accretion are considered to proceed in two steps with attachment to a surface followed by cell-to-cell adhesion. This is followed by maturation of the biofilm that frequently is represented by stratification of bacteria within the biofilms and finally dispersal from the sessile community. For this process to occur, it would be expected that environmental conditions impact the type of bacteria and their ability to survive and replicate within a mutualistic complex. The success of this would require regulation of a range of genes specific for biofilm life versus those required for a successful planktonic existence. Biofilms have been shown to have a high persistence once the infections are established and thus responsible for many chronic infectious processes. This related directly to their phenotypic resistance to high concentrations of antimicrobials [13,17], and ability to modulate the host immune systems [18,19].

Numerous studies have been conducted on biofilms of *S. aureus* due to its major role in infections of catheters and prosthetics. These studies have identified genetic regulation and molecular components involved in biofilm formation and maturation [20]. Similar types of studies were reported on novel biofilm gene regulation in *M. tuberculosis* [21,22]. An additional major feature of biofilms is the altered microenvironment that is created within and surrounding the development of the biofilms. Characteristics of these differences include both structural and physiological. The exracellular matrix accumulates and often encases the multicellular structures within the 3-D architecture of the biofilms. With *Y. pestis* the extracellular matrix of the biofilm contains a homopolymer of N-acetyl-D-glucosamine, which is a constituent of many bacterial biofilms and may adversely affect both host resistance mechanisms and extrinsic antibiotic administration. This extracellular polymeric substance has been reported to facilitate tolerance to environmental stresses, and contributes to physiological adaptation of individual bacterial cells in the heterogeneous microenvironments within the complex architecture of biofilms [23]. The physiological adaptation studies have identified available nutrients, temperature, and pH as factors that can contribute to the altered microbial ecologies in biofilms. Studies with *P. aeruginosa, Klebsiella pneumoniae*, and *V. cholera* have shown that increased pH leads to a higher biofilm production *in vitro* [24]. Similar studies examined the effect of pH on production of extracellular virulence factors of *P. aeruginosa* growing on catheter biofilms. Both alginate and proteinase production was higher at pH 8; in contrast, siderophores (pyochelin and pyoverdin) that were synthesized to a greater level at pH 5 [25]. It has also been suggested that the pH at the site of infection is one of a number of factors that may significantly influence the *in vivo* activity of an antibiotic prescribed for treatment of infection. Results by Moriarty et al. [26] showed that growth in an acidic environment might be expected to reduce the susceptibility of *P. aeruginosa* to certain antibiotics. A substantial decrease in the biofilm production was observed at 37 °C compared with the amount of biofilm formed at 30 °C with most strains of these species. Studies of *S. aureus* and *Salmonella typhimurium* biofilms demonstrated significant differences in gene expression in biofilms cultivated at different acidic, neutral or alakaline pH values [27]. A number of these genes have been directly related to virulence of these pathogens. As an additional environmental regulator of biofilms, data from cystic fibrosis would suggest that the *P. aeruginosa* biofilms likely form under anaerobic conditions [28]. This tenet can be extrapolated as both anaerobically grown planktonic bacteria and biofilm bacteria were significantly less susceptible to single and combination antibiotics compared to aerobic growth of planktonic bacteria. Additionally, antibiotic combinations that killed under anaerobic conditions frequently differed from those that were bactericidal against the same microorganisms grown as biofilms [29].

The vast majority of studies of medically significant pathogens have used bacteria in the planktonic state, although many express their virulence through the formation of biofilms. More recent *in vivo* and *in vitro* studies have documented innate and adaptive immune responses to biofilms. However, strategies used by bacteria in biofilms to resist/minimize various immune responses have also been demonstrated. Hence, with biofilm infections often present as persistent infections with simultaneous activation of both innate and adaptive immune responses can be detected. Unfortunately, in many cases, neither of these response arms can eliminate the biofilm pathogen, but frequently, as noted in periodontitis, causes collateral tissue damage.

It has been shown that *S. aureus* biofilms induce a distinct inflammatory response compared to their planktonic counterparts. The differential gene expression and production of inflammatory cytokines by the biofilm was predicted to have an adverse effect on the formation and persistence of chronic wounds [30].

Furthermore, *S. aureus* biofilms significantly reduced keratinocyte viability and significantly increased apoptosis compared with planktonic bacterial cells [31]. With *S. aureus* biofilms PMNs moved across the biofilm and took up bacteria as they moved, while with a related pathogen, *Staphylococcus epidermidis*, the PMN were rather immobile, and phagocytosis was limited to bacteria closely associated with the surface of the PMNs. Consequently, it was interpreted that *S. aureus* biofilms are more sensitive towards PMN attack compared to *S. epidermidis*, and that these types of biofilms are not inherently protected against the attack by phagocytic cells, albeit the inherent sensitivity is related to bacterial species or strains [32]. A separate study showed that opsonization of *S. aureus* biofilms with human serum IgG binding resulted in complement activation. However, this immune process did not affect the adherence of PMN to the biofilms nor did it enhance degranulation or phagocytosis [33]. Finally, it was found that developing "young" biofilms of *S. aureus* were more sensitive towards attack by PMNs compared to mature biofilms [34]. Thus, studies developing data regarding how host protective features and host cells interface with biofilms need to consider substantive differences between newly developing versus mature biofilms with respect to clearly elucidating how host cells try to manage biofilm accretion.

Biofilms of *P. aeruginosa* induced a higher production of TNFα and IL-6 than planktonic bacteria [35]. In addition to the cytokine levels, reactive nitrogen species, and stimulated macrophage secretory products were generated in greater levels with biofilms of