Revista Clínica de Periodoncia, Implantología y Rehabilitación Oral
ISSN: 0718-5391
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Sociedad de Periodoncia de Chile
Chile

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Revista Clínica de Periodoncia, Implantología y Rehabilitación Oral, vol. 2, núm. 3, diciembre, 2009, pp. 187-192
Sociedad de Periodoncia de Chile
Santiago, Chile

Literature Review

Subgingival Biofilm Communities in Health and Disease

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ABSTRACT

Oral biofilm-related diseases such as periodontal disease are infection processes that arise from the resident (indigenous) flora. Prior to the development of a periodontal lesion, a change in the proportion of certain species with greater pathogenic potential occurs within the biofilm. This change from a "commensal" flora to one considered pathogenic is accompanied by a disruption of the immune homeostasis and development of an inflammatory response. Chronic inflammation of the supporting periodontal structures eventually progresses to tooth loss. Although periodontal diseases have a multifactorial etiology in which environmental and host factors play an important role, polymicrobial biofilm communities with pathogenic properties are the primary etiological factor of periodontal lesions. Therefore, a thorough understanding of the events that lead to the maturation of subgingival biofilm communities is necessary in order to develop better diagnostic and treatment strategies. This review article will summerize our current understanding of the ecology of subgingival biofilms and the role of these multi-species communities as etiological agents of periodontal disease. An overview of the process of subgingival biofilm formation will be presented followed by a description of the ecological determinants of biofilm development in the subgingival environment. Finally, the concept of subgingival polymicrobial biofilm communities as the etiological agents that initiate a host-mediated inflamamtory response will be discussed.

Rev. Clin. Periodoncia Implantol. Rehábil. Oral Vol. 2(3); 187-192, 2009.

Key words: Subgingival biofilms, microbial communities, periodontal disease etiology.

INTRODUCTION

Periodontal diseases are the result of an infection process in which microorganisms accumulate on the surfaces of teeth and adjacent supporting periodontal structures. The non-shedding nature of the tooth provides a unique opportunity for microorganisms to accumulate and form thick deposits historically refered to as *plaque*. However, it is now evident that these deposits constitute an organized structure in which microorganisms interact metabolically and coexist as a community; hence the current preferred terminology for dental plaque is *biofilm*. Undisturbed microbial accumulation allows the maturation of the biofilm over time, a process driven by shifts in the predominant bacterial species present in the biofilm[1]. Some of the late-colonizing species of oral biofilms display greater pathogenic potential than the predominant species during early plaque formation[2]. The inability of the host to contain the proliferation of these late-colonizing pathogenic bacteria results, in the susceptible host, in a chronic disease process that ultimately leads to destruction of the supporting structures of the tooth.

It is estimated that about 60% of the oral microflora has not been cultivated yet[3,4]. Of the 40% that has been cultivated, about ten microbial species have been recognized to have pathogenic potential in periodontitis[5-8]. Most of these species are Gram-negative anaerobic bacteria that flourish in subgingival biofilms where oxygen tension and redox potential are low[9,10] and where the gingival crevicular fluid serves as a source of nutrients[11,12]. The species attributed with the greatest pathogenic potential in periodontitis, which have also been the focus of most studies, include *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*[7,13,14]. Other Gram-negative species such as *Fusobacterium nucleatum, Campylobacter* sp. or *Prevotella* sp. are believed to preceed and facilitate the colonization of the more virulent species mentioned above and may also contribute to activate an immune response that results in irreversible tissue damage[2,7,15,16]. However, little information is available on that 60% of the uncultivated flora that could potentially harbour species with similar pathogenic potential.

It is now recognized that the presence of a bacterial consortium composed of certain combination of species, rather than the presence of single species, may be more indicative of the risk to develop periodontal disease[7,17]. The survival of pathogenic species in mature oral biofilm consortia is thought to be regulated by physical and metabolic interactions within community members[1,18]. These interactions occur at several levels including physical contact, metabolic exchange, small signal molecule-mediated communication and exchange of genetic material[19]. It is important to understand that in these complex biofilm communities, the mere presence of a single organism is not indicative of the composition and metabolic properties of the whole community. Similarly, the host response to the whole community is likely to be different than the response to each individual organism. Therefore, the study of microbial pathogenesis in relation to periodontal disease has shifted focus from the study of single bacterial species to the study of the ecology and virulence of subgingival polymicrobial communities.

DEVELOPMENT OF SUBGINGIVAL BIOFILMS

Early biofilm formation. Immediately after tooth brushing specific salivary glycoproteins are adsorbed onto the tooth surface forming a film called *acquired pellicle* (Figure 1). This host-derived pellicle is composed of mucins, agglutinins, proline-rich proteins, phosphate-rich proteins such as statherin, and enzymes such as alpha-amylase[20]. Different pellicle components act as receptors for particular oral species. Microorganisms rapidly start colonizing this pristine pellicle, a process termed *adhesion*. With the attachment of each new cell type, a nascent surface is presented for the attachment of other kinds of bacteria, a process termed *co-adhesion*[21,22]. Bacterial cells also have the ability to form multi-species aggregrates in suspension, a phenomenon named *co-aggregation*[23]. Coaggregation is defined as the specific cell-to-cell recognition that occurs between genetically distinct cell types. Each cell type bears on its surface one or more kinds of coaggregation mediators, which serve as either adhesins (protein) or receptors (polysaccharide). During coaggregation, adhesins on the surface of one cell type recognize and bind to a complimentary polysaccharide on the surface of another cell type[24,25,26]. Coaggregation is a specific event in the sense that each bacterial cell has the potential to interact only with specific coaggregation partners. These partnerships are central to the development of supragingival and subgingival plaque (Figure 2).

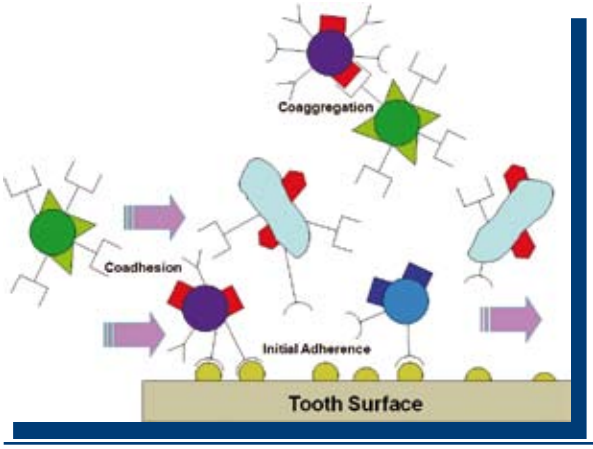

Figure 1. Diagrammatic model of initial colonization and the role of coaggregation and coadhesion in establishing a mixed-species community of oral bacteria on acquired pellicle-conditioned enamel surface. Bacteria are represented by circles (e.g. *Streptococcus spp*.) or oblong shapes (e.g. *Actinomyces spp*.). Each bacterial cell bears several functional structures on its surface. Structures in solid colors represent receptor polysaccharides. Complementary structures borne away from the surface by a stem represent fimbriae tipped with adhesins, which recognize cognate

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Trabajo recibido el x/xx/2009 / Aprobado para su publicación el xx/xx/2009.

receptor polysaccharides. An example of this complementary fit is shown in the upper right. Adhesins in the salivary acquired pellicle (yellow circles) also recognize receptors. The acquired pellicle contains a wide variety of receptors including highly glycosylated glycoproteins, proline-rich proteins, proline-rich glycoproteins, sialylated mucins, salivary agglutinin, alpha-amylase, histatins, cystatins, and statherins.

Bacteria bearing adhesins depicted as a semi-circle are capable of adhering to the acquired pellicle (initial adherence), but other bacteria bearing non-complimentary adhesins must bind to an already adhered cell (coadhesion) or one in suspension (coaggregation) to be able to colonize the tooth surface.

Salivary flow (depicted by arrows) creates an environment that necessitates adherence of cells to prevent being washed out of the pioneer community.

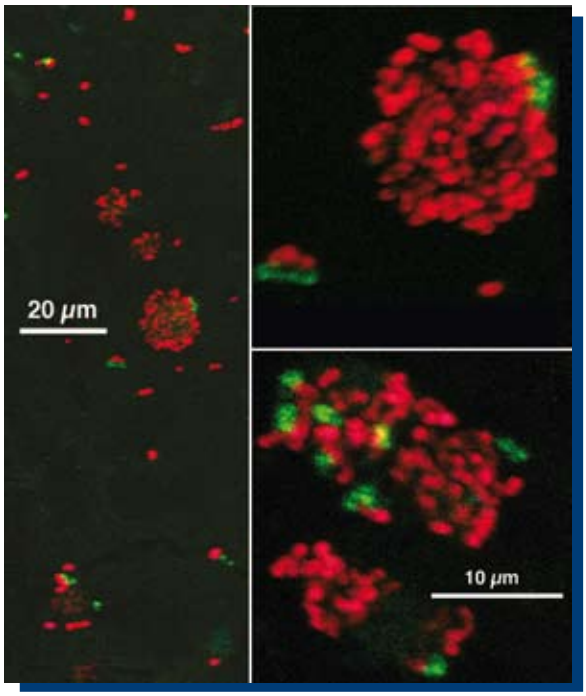

Figure 2. Coaggregation-mediated interactions in dental plaque biofilms formed in vivo on retrievable enamel chips worn by a volunteer for 8 hours. Two highly specific and fluorescently conjugated antibodies were used to identify the coaggregation mediators on bacterial cells in situ. Cells were stained with an antibody against type 2 fimbriae of *Actinomyces naeslundii* (green) and an antibody against complimentary receptor polysaccharide (RPS) of *Streptococcus oralis* (red). It is known from coaggregation surveys that type 2 fimbriae carry the adhesin that recognizes streptococcal receptor polysaccharide and that fimbriae-less mutants of *A. naeslundii* do not coaggregate. Low magnification view of enamel surface (left panel). Electronic zoom of central region of left panel (upper right panel) and of a different location on the enamel (lower right panel). In all panels, note the intimate interaction between anti-type-2-fimbriae-reactive cells and anti-RPS-reactive cells. From Palmer et al. (2003)[76].

The process of biofilm formation is driven by microbial succession events in which some genera colonize nascent surfaces followed by the arrival of secondary and late-colonizing species. Several studies have identified streptococci as the predominant colonizers of early enamel biofilms[27,28,29]. Nyvad and Kilian (1987)[29] characterized the cultivable microflora colonizing enamel pieces exposed to the oral cavity. Streptococci were shown to compose about 63% (mean value of samples from 4 individuals) of bacteria isolated after 4 hours of plaque formation and 86% of bacteria isolated after 8 hours. A variety of other bacteria such as veillonellae and actinomyces were also cultivated. The era of molecular biology opened the possibility to characterize bacterial communities with cultivation no longer a requirement. Li *et al.* (2004)[28] used the "checkerboard" DNA-DNA hybridization technique[30] to investigate early supragingival plaque of 15 healthy individuals. Fourty DNA probes were used, each containing the signature sequence of the 16S ribosomal RNA (rRNA) gene for one bacterial species, in order to analyze samples of supragingival plaque collected after 0, 2, 4 and 6 hours of plaque formation. This study identified *Streptococcus* sp., in particular *S. mitis* and *S. oralis*, as being the predominant early colonizers, increasing in numbers especially after 4 hour of biofilm formation. Other identifiable and moderately present species were *Actinomyces naeslundii, Streptococcus gordonii, Eikenella corrodens* and *Neisseria mucosa*. Also of interest was the detection of low levels of the periodontal pathogens *Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola* and *Aggregatibacter (Actinobacillus)* actinomycetemcomitans at this early stage of biofilm formation. A recent study by our group[27] characterized the initial microflora by use of 16S rRNA gene sequencing, which allows identification of the entire range of species present in a sample. This study utilized retrievable enamel chips, which were placed in the oral cavity of volunteers for 4 and 8 hours. Bacteria were released from the enamel chips by sonication and the 16S rRNA genes of the bacteria in the sample were amplified by the polymerase chain reaction (PCR). 16S rRNA gene libraries were constructed for 3 subjects sampled at 4 hours and 8 hours of colonization of the enamel chips. It was observed that the initial communities from all subjects were dominated by *Streptococcus* spp. belonging to the *S. oralis/S. mitis* group. Sequences were grouped as phylotypes, which were defined as those sequences similar to one another by 98%. The most abundant phylotypes, apart from those classified as streptococci, belonged to the genera *Actinomyces, Gemella, Granulicatella, Neisseria, Prevotella, Rothia, Veillonella* and also included uncultured species from the class *Clostridia*. It was also interesting that the initial communities of some subjects contained Gram-negative anaerobic bacteria such as *Prevotella* spp. and *Porphyromonas* spp. confirming that anaerobic periodontopathogens can colonize early biofilms. Importantly, this study found that the microbial composition, even at this early stage of plaque development, appeared to be unique to each subject. Thus, early dental plaque microflora appears to vary on a subject-specific basis.

Subgingival plaque is probably formed by the spread of supragingival plaque down into the gingival sulcus[31]. The study of initial subgingival biofilms represents a greater challenge due to the lack of models that mimic subgingival colonization or allow appropriate sampling. Quirynen and collaborators[32,33] tried to overcome this limitation by analyzing the subgingival microflora present in so called "pristine" pockets, which were created after insertion of transgingival abutments in previously submerged dental implants. This microbiologically "pristine" pockets had depths of 2.5 to 6 mm and were sampled after 1, 2 and 4 weeks of abutment connection. Each site sampled was analyzed for the presence of 40 bacterial species using DNA-DNA checkerboard hybridization. After 1 week of subgingival biofilm development, the species detected in the highest quantities were *Neisseria mucosa, Actinomyces naeslundii, Veillonella parvula* and *Streptococcus gordonii*, microorganisms known to be abundant in early supragingival enamel biofilms[27]. Furthermore, it was also reported that low counts of periodontopathogens were seen at early stages of subgingival plaque development. These results highlight the similarities between the processes of subgingival and supragingival biofilm formation. Moreover, periodontopathogens were more commonly found when other teeth in the dentition of the sampled individual also harbored them. The authors then suggested that the colonization of pristine pockets around implants occurs from the bacteria present in saliva (representing the microbial load in the remaining dentition) that accumulates first supragingivally and then subgingivally around the newly placed implants[32]. A follow-up study confirmed these findings and reported that the initial colonization of peri-implant pockets with bacteria associated with periodontitis occurs within 2 weeks[33]. This evidence strongly supports the concept that subgingival plaque development is directly influenced by the supragingival environment. Other data are in agreement showing that just by controlling the supragingival microbial load through scaling and oral hygiene, the proportion of subgingival Gram-negative anaerobic bacteria in moderate periodontal pockets is decreased[34].

Bacterial succession and increase in diversity in mature biofilms.

If undisturbed, the bacterial mass in plaque biofilms will continue to increase due to a combination of the continued growth of adherent organisms, the coadhesion of new cell types and the synthesis of extracellular polymers. Early colonizers are generally facultatively anaerobic bacteria but as biofilm thickness increases and bacterial metabolites with low oxidation-reduction potential accumulate, an oxygen gradient is believed to form that allows organisms less resistant to oxygen, especially obligate anaerobes, to proliferate. Therefore, over time, the composition of plaque biofilms changes to include more Gram-negative anaerobic species. After seven days of undisrupted biofilm accumulation the bacterial population has been documented to shift from the initial Gram-positive cocci to predominantly rods and filaments[35] with the appearance of spirochetes and vibrios[36]. A classic study by Ritz (1967) described that changes occur in the microbial composition of supragingival plaque over an observation period of 9 days. Facultative anaerobes and aerobes from the genera *Streptococcus* and *Neisseria* predominated at day 1 of plaque formation, while after 9 days a shift occurred and the proportions of these genera decreased and *Veillonella, Corynebacterium* (now probably considered to be *Actinomyces*) and

Fusobacterium increased. These shifts in the microbial composition of plaque have great significance as they correlate with the appearance of gingival inflammation. The experimental gingivitis studies conducted by Löe and coworkers[36] demonstrated that the shift from a cocci-dominated plaque to one composed of cocci, rods, filamentous organisms, vibrios and spirochetes correlated with the development of gingivitis. A later study by Listgarten (1976)[37] described the ultrastructural characteristics of mature plaque present on extracted teeth sorrounded by either healthy periodontal tissues or tissues with various degrees of periodontal disease. This study revealed that the health-associated microbial biofilms consisted of a thin layer of adherent bacterial cells with the characteristics of Gram-positive cocci. In contrast, the samples from teeth with gingivitis contained a greater variety of Gram-negative and Gram-positive morphotypes, including coccoid and filamentous forms. Diversity further increased in the samples from teeth with chronic periodontitis which contained a dense, predominantly filamentous supragingival plaque and a subgingival component containing flagellated bacteria, spirochaetes and small Gram-negative bacteria. These early studies demonstrated that the maturation of plaque is accompanied by changes in the predominant bacterial species and increased bacterial diversity. They also established a cause-effect relationship between these temporal changes in the microflora and the appearance of inflammation.

Subgingival biofilms associated with periodontitis.

Mature oral biofilms appear to be a very diverse community of species. Furthermore, in subjects with periodontitis the diversity of the subgingival resident organisms seems higher than in healthy subjects. A review of the literature available until 1994[6] found evidence to consider approximately a dozen microorganisms as possible periodontopathogens. These microorganisms included *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Tannerella forsythia, Campylobacter rectus, Eikenella corrodens, Peptostreptococcus micros, Selenomonas* sp., *Eubacterium* sp., *spirochetes* (such as *Treponema denticola*) and *Streptococcus intermedius*. The evidence for *Aggregatibacter actinomycetemcomitans* as an etiologic agent in aggressive forms of periodontal disease was also reviewed and found to be strong. The World Workshop in Periodontology in 1996 (Consensus Report 1996) reviewed the body of evidence for all these microorganisms and concluded that at the time only *A. actinomycetemcomitans, P. gingivalis* and *T. forsythia* could be conclusively classified as periodontal pathogens. A later study by Socransky *et al.* (1998)[7] found strong evidence to link the red complex organisms (*P. gingivalis, T. forsythia* and *T. denticola*) with presence of disease. However, we know from studies like that of Paster et al. (2001)[4] that approximately 415 species exist in subgingival plaque. Of these species, approximately 40% remain uncultured. Therefore, it is likely that new periodontal pathogens might emerge from the group of uncultured bacteria.

It seems also necessary to consider that not all bacteria present in sites with periodontitis may be actively involved in the transition from health to disease. Virulent bacteria may not be able to colonize a site until the microflora has shifted and the inflammatory process has initiated. The role of many microorganisms considered periodontopathogens might be to perpetuate the imbalance in the microflora and therefore the inflammatory response induced by other bacteria. Due to understandable methodological limitations, very few studies have tried to characterize the microflora at the precise moment the shift from health to disease occurs. Tanner *et al.* (1998)[8] studied the bacterial species associated with the initial development of a periodontal lesion. This study is one of the few longitudinal studies to identify the organisms associated with the shift from health to disease. Their data suggest that T*annerella forsythia, Campylobacter rectus* and *Selenomonas noxia* are the major species that characterized sites converting from health to disease. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were detected infrequently in this population, which suggests they might populate subgingival biofilms later during the disease process. It is also evident that subject-specific factors make it very difficult to categorize a microorganism as causative of disease. The same organism might not display the same virulence potential in all subjects, as the interaction with other bacteria could condition its growth and gene expression. Similarly, one microorganism might have low pathogenic potential in one subject while in another subject, with a different genetic background, the presence and proliferation of this bacterium might create an unfavourable immune response, as it is known that host factors greatly influence disease susceptibility[38,39,40]. Thus, our undertanding of pathogenic subgingival biofilm communities will continue to evolve hand in hand with molecular techniques that will allow a better characterization of communities at different disease states.

ICROBIAL INTERACTIONS IN SUBGINGIVAL BIOFILMS

Mature dental plaque is considered to be a climax biofilm community, which is defined as a stable community in equilibrium with its environment, remaining reasonably stable over time and reflecting a dynamic situation in which microbial cells are continuously dying and being replaced. Theoretically, the climax biofilm community will reproduce itself once eliminated if the same environmental conditions occur[41]. Microorganisms within the climax biofilm community have the ability to interact with each other via communication signals sent from cell to cell[42], establishing food chains or other types of metabolic cooperation that maintain the stability of the community over time. A classic example of this type of interactions among oral microorganisms is that provided by the food chain between streptococci and *Veillonella* sp. Streptococci ferment carbohydrates to form lactic acid. Veillonellae use lactic acid as a preferred fermentation substrate. In an *in vitro* biofilm model, these two microorganisms have also been shown to communicate via small difusible signals that mediate changes in gene expression profiles in one organism when the other one is present[43]. Distance between the sender and receiver of the signal appears to be critical in that only those streptococci in direct contact (coaggregated) with veillonellae showed changes in gene expression[43]. Events of this type might be of common occurance *in vivo* in oral biofilms directly influencing community stability.

An example of nutritional interdependence between periodontal pathogens is that described to occur between *Porphyromonas gingivalis* and *Treponema denticola*. When grown in coculture, *P. gingivalis* metabolizes succinate produced by *T. denticola* which in turn metabolizes isobutyric acid excreted by *P. gingivalis*[44]. This interaction represents metabolic mutualism (benefitial for both organisms) since *P. gingivalis* and *T. denticola* show enhanced planktonic and biofilm growth as a co-culture in comparison to mono-species growth. Indeed, it is very likely that such an interaction occurs in vivo as Kigure et al. (1995) has demonstrated that *P. gingivalis* and *T. denticola* co-exist in periodontal pockets.

Cooperative metabolic interactions could also occur when one species modifies the local microenvironment making it more suitable for the growth of another species. For example, *Fusobacterium nucleatum* and *Prevotella intermedia* can generate significant amounts of metabolites such as ammonia that rise the pH and may make the environment more suitable to species with less acid-neutralizing potential such as *P. gingivalis*[45]. Moreover, our group has also demonstrated that *F. nucleatum* contributes to generating a reducing and capnophilic environment that is necessary for the growth of *P. gingivalis*[46]. Using continuous co-culturing techniques we demonstrated that *F. nucleatum* was able to support the growth of *P. gingivalis* in aerated and CO_2-depleted environments, conditions in which *P. gingivalis*, as a continuous monoculture, was not able to survive.

The specific interdependence (physical and metabolic) of different bacterial species might be a key factor that determines whether, once disturbed, a community of microorganisms will re-establish itself in a similar organized manner to the way it existed previously. This is the challenge faced by periodontal therapy. The elimination of the etiologic factors gives the host an opportunity to recover but if adequate oral hygiene is not performed by the patient, the climax community will tend to re-establish and harbour similar species as those encountered before therapy[41,47].

OTHER ECOLOGICAL DETERMINANTS OF SUBGINGIVAL BIOFILMS

Subgingival plaque is believed to be an organized community with different spatial niches created by the two different surfaces available for attachment of microorganisms, i.e., the tooth and the sulcular epithelium. In addition, a gradient of oxygen occurs between the surface and bottom of the pocket[10]. Different available niches contribute to the non-random organization of bacteria in the periodontal pocket. A study by Kigure and coworkers (1995) found that *Treponema denticola* inhabits the surface layers of subgingival plaque in moderately deep pockets, while *Porphyromonas gingivalis* is predominantly seen beneath them. In deeper pockets both bacteria co-existed. A possible explanation for this distribution is that *T. denticola* may be more aerotolerant than *P. gingivalis* and able to proliferate in the outside layer of moderate pockets, while *P. gingivalis* is restricted to the inner layer. In deeper pockets where the oxidation-reduction potential is lower, both bacteria can flourish in all layers. Noiri and coworkers[48,49] also showed that the localization of certain bacterial species differs in subgingival plaque. *Prevotella nigrescens* and *Porphyromonas gingivalis* tend to be located at the epithelium-associated plaque in the middle pocket zone while species such as *Campylobacter rectus, Treponema denticola* and *Fusobacterium nucleatum* were found

in both the middle and deep pocket zones; *C. rectus* was located in both unattached and attached plaque and *T. denticola* and *F. nucleatum* located mainly in the unattached plaque. Thus, the metabolic requirements of a microorganism as well as other properties such as its tolerance to oxygen, adhesion to host surfaces, coaggregation with other bacteria and motility determine the areas within the periodontal pocket in which proliferation of those bacteria can occur.

It is important to understand that the composition of the microflora at a given moment might also be influenced by the host. The undisturbed accumulation of plaque produces gingivitis. The increase in the gingival crevicular fluid (GCF) and bleeding will in turn facilitate the proliferation of certain Gram-negative anaerobic genera such as *Porphyromonas* and *Prevotella* that require iron stored in host proteins such as haemoglobin and transferrin for growth. The proliferation of these genera will in turn exacerbate the immune response and increase the clinical signs of inflammation[50]. Other host factors such as cytokines and antimicrobial peptides may also modulate biofilm development, however, this remains an area largely unexplored.

HOST RESPONSE TO POLYMICROBIAL BIOFILMS

Growth as a biofilm has been shown to modulate gene expression of microorganisms conferring them different virulence attributes than their planktonic counterparts[51,52,53]. Moreover, microbial gene expression is also modulated by co-existence of microorganisms in polymicrobial communities[43,54,55,56,57]. However, most *in vitro* and *in vivo* studies of the host response to oral microorganisms have been carried out with single microbial species, grown as a suspension in liquid laboratory medium, in conditions that may not reflect the physiologic state of microbes in the oral environment[58,59,60,61]. In limited cases the effects of co-infection with several microbial species on the immune response have been determined[62,63,64]. Moreover, most *in vitro* studies of the response of cells to specific microbial species have been done by comparing infected cells to uninfected cells. Although, these experiments have been very useful in determining the response of host cells to a specific microbial challenge, they do not reflect the state of cells during health or disease. In no instance is the oral mucosa devoid of microorganisms. Health is characterized by a state of homeostasis with a commensal polymicrobial biofilm, while disease is characterized by the response to a pathogenic biofilm community.

There is enough evidence to support the notion that those species regarded as commensal trigger a different response in host cells than those considered pathogenic[2,58,65]. Transcriptional profiling of an epithelial cell line infected with either *Streptococcus gordonii, Fusobacterium nucleatum, Porphyromonas gingivalis or Aggregatibacter actinomycetemcomitans* revealed that these bacteria affect common gene pathways but in a way that is specific to each microorganism[2]. The same study also observed that *F. nucleatum* or *S. gordonii*, considered commensals, perturbed the transcriptome much less significantly than *P. gingivalis* or *A. actinomycetemcomitans*, considered pathogens. Furthermore, it was possible to discriminate between pathogens and commensals by the cytokines that were differentially regulated in epithelial cells. Oral commensal and pathogens have also been shown to differentially regulate antimicrobial peptides[16] and utilize different pathways for their induction in epithelial cells[66]. However, the response of oral tissues to infection with different microbial consortia remains largely uncharacterized. It is likely that organisms within polymicrobial biofilms trigger antagonistic responses by the host and that pre-exposure with a certain microbe may affect the later response to a different microbial challenge[58,67]. Similarly, the co-existence of different microbial species within a biofilm could enhance their virulence potential as demonstrated by *in vitro*[68,69] and animal studies[63,70,71]. Therefore, the characterization of the host response to commensal and pathogenic oral biofilms is needed as a first step in dissecting those host response mechanisms that might be important during periodontal disease processes. Furthermore, therapeutic approaches to combat periodontal diseases should be based in their polymicrobial etiology rather than focusing on single microorganisms.

A LOOK TO THE FUTURE

Our current understanding of the pathogenesis of periodontal disease has emerged from studies spanning several decades which collectively support the bacterial biofilm as the primary etiologic factor of the disease. More importantly, the host does not respond equally to all the components of the biofilm. A selected group of organisms is considered to have pathogenic potential, while the rest of the biofilm components are considered commensals. The studies that have elucidated the virulence potential of periodontopathogens have been generally carried out using monocultures of bacteria. It is important to remember that the pathogenic potential of one species might be modified by the presence of another species since the host responds differently to organisms forming part of a mixed culture as compared to monocultures[63]. More importantly, the genetic background of the host has been shown to play a role in disease susceptibility[39]. Therefore, the etiology of periodontal disease is likely not related to a single species of bacteria but it is the result of multi-bacterial profiles as they relate to the genetic background of the host.

Current molecular biology techniques have revealed the great diversity of bacterial species present in subgingival plaque, of which we have only been able to culture about 60%[4]. A few studies have already used the 16S rRNA gene sequences of uncultured bacteria to assess their presence in plaque samples of subjects with periodontitis. Brinig *et al.*(2003)[72] evaluated the prevalence of the uncultured division TM7 in human subgingival plaque and determined its association with disease. This study found that sites with mild periodontitis harboured significantly higher proportions of TM7 than healthy sites or sites with moderate and severe periodontitis. Kumar *et al.* (2003)[73] used PCR to detect 39 species in 66 subjects with periodontitis and 66 healthy subjects. This study found an association between periodontitis and species from the phyla *Deferribacteres, Bacteroidetes, Megasphaera*, TM7 and OP11. A more recent study by the same group (Kumar et al. 2006)[73] analyzed, by 16S rRNA gene amplification and cloning, the overall plaque composition of samples obtained from patients whose periodontal health improved, was stable or worsened over a 2 year period. They found phylotypes from the genera *Treponema, Dialister, Selenomonas* and the species *Filifactor alocis* to be associated with deterioration of the periodontal status. *P. gingivalis* and *T. forsythia* were rarely detected and constituted only a minor component of the plaque in all sites. In agreement with other studies[27], it was observed that samples varied greatly among subjects which supports current thinking that bacterial communities from different subjects differ significantly. Furthermore, it is evident from most studies that a shift in the whole bacterial community should be considered the main etiologic factor related to disease rather than the increase or decrease of a single species. Future studies should assess comprehensive bacterial profiles coupled with analysis of the genetic background of the host and changes in clinical parameters in order to provide a more definitive understanding of which subjects are at risk for disease progression.

Moreover, it has also become evident that bacterial species are in fact heterogeneous populations about which it is not always possible to generalize, particularly with regard to pathogenic potential[74]. Each bacterial species is composed of a number of clones that might differ in their virulence properties. Each individual carries a limited number of these clones, which are very stable during the lifetime of the individual. A clear example of population heterogeneity with regard to virulence potential is seen in *A. actinomycetemcomitans.* A specific clone of this species, the JP2 clone, has been consistently isolated from individuals of North and West African descent suffering from aggressive periodontitis[75]. The JP2 clone shows elevated leukotoxin activity due to increased expression of the *ltx* gene. Poulsen *et al.* (1994) showed this increased expression of *ltx* is the result of a 530 bp deletion in the promoter region of the gene. Haubek *et al* (2001) demonstrated an association between the presence of this clone and aggressive periodontitis but not between the presence of *A. actinomycetemcomitans* (the total bacterial population) and aggressive disease. It is then evident that attempts to identify etiologic agents of oral diseases by searching for association between the presence of particular bacteria and disease activity are all but too simplistic. Thus, we are in urgent need of refining our knowledge on bacterial population structures and possible presence of specific clones with increased virulence potential within a single strain.

Some studies have also pointed out that viruses could be possible contributors to the etiology of periodontal disease. Kamma *et al.* (2001) reported that human cytomegalovirus (HCMV), Epstein-Barr virus type 1 and herpes simplex virus (HSV) were detected with higher frequency in actively progressing sites as compared to stable periodontitis sites in a population with aggressive periodontitis. Furthermore, Ling *et al.* (2004) found a positive association between herpes viruses and clinical parameters of periodontal disease severity. Saygun *et al.* (2004) reported positive associations between the presence of HCMV and HSV

and *P. gingivalis, P. intermedia, T. forsythia* and *C. rectus*. A mechanism to explain this possible inter-relationship might be that infection of host tissues with a viral agent may impair local host defenses allowing periodontal pathogens to proliferate. Further research is necessary to clarify this possible viral-bacterial coinfection relationship.

CONCLUDING REMARKS

Periodontal diseases have a multifactorial etiology in which biofilm communities with pathogenic properties override the protective immune mechanisms of the host leading to the destruction of the supportive periodontal structures. Humans harbour distinct subgingival biofilms that represent those microorganisms that colonized and adapted to a particular host. If undisrupted, subgingival biofilms evolve from early communities with a limited number of species to complex mature entities with great species diversity. The maturation of these communities is regulated by physical and metabolic interactions with other members of the community. These interactions might promote flourishing of bacteria considered to have greater pathogenic potential. These pathogens have greater ability to disrupt or exacerbate host immune responses than the socalled commensals. However, the survival of these periodontal pathogens is supported by other community members; thus the multi-species community as a whole is responsible for disease initiation and progression.

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