2849 Periodontopathogenic Bacterial Biofilms and Planktonic Cells - An Ultrastructural Comparison.

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Dental plaque, the aetiologic agent of both dental caries and chronic inflammatory periodontal disease, is a bacterial biofilm formed by adherence of planktonic free-living bacteria to a surface and their subsequent proliferation. Much past research has involved the assessment of bacterial death in planktonic culture with a view to arresting the advance of plaque *in vivo*. However it is now apparent that the behaviour of members of a biofilm, in particular their resilience to adverse conditions differs from that of planktonic cells. In this study we cultured *Actinobacillus actinomycetemcomitans* in Wilkins Chalgren broth with 10% defibrinated horse blood and as a biofilm (on cellulose nitrate membrane filters on Wilkins Chalgren agar plates) for 24, 48 and 72 hours. Resulting cultures were fixationed with glutaraldehyde, post-fixed in osmium tetroxide, dehydrated and embedded in LR White resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL 100CX transmission electron microscope. Although there was progressive alteration in the appearance of both protoplasm and cell wall with time, the rate of change was faster for cells in broth as opposed to biofilm. Planktonic bacteria showed intact cells at 24hrs but by 72hrs there was advanced lysis and protoplasmic condensation. However biofilm cells displayed only slight lysis and little protoplasmic condensation at 72hrs. Planktonic cells displayed surface-associated fibrils at all time periods though those in biofilm only did so at 72hrs. These differences in ultrastructure between planktonic and biofilm bacteria are evidence of the slower growth rate of cells in biofilm which in turn may account for the refractory nature of biofilms.

2850 Resistance of *Porphyromonas gingivalis* in biofilms to doxycycline and metronidazole

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Bacteria living in biofilms often exhibit increased resistance to antibiotics. The purpose of the present study was to determine the susceptibility of *Porphyromonas gingivalis (Pg)* in biofilms to doxycycline and metronidazole. 48h biofilms of three reference strains and three clinical isolates of *Pg* were established on membrane filters in a Modified Robbins Device (MRD) under anaerobic conditions, yielding about 10^7-10^8 cells/filter. After removal of planktonic bacteria by rinsing, the filters were transferred to tryptic soy agar plates (TSA, Difco) containing doxycycline or metronidazole (Sigma) in two-fold dilutions from 0-128 µg/ml for determination of the minimal inhibitory concentration (MIC). 10^4, 10^7 and 10^9 cells of planktonic bacteria of the same strain and a reference strain were inoculated, too. After incubation for 7d non-growing bacteria were transferred to drug-free TSA for determination of the minimal bactericidal concentration (MBC). The MIC for planktonic bacteria (10^4 cells) was 0.125-0.25 µg/ml of doxycycline and 0.063-0.125 µg/ml of metronidazole. The MIC of doxycycline to biofilm bacteria was above this level, but was similar for 10^7 planktonic bacteria. The MBC of doxycycline to biofilm bacteria increased up to more than 100 times above the biofilm MIC, while a minor increase was seen for planktonic cells. The MIC of metronidazole to biofilm bacteria increased, too. The MBC of metronidazole to biofilm cells was generally twice the biofilm MIC-value. The MIC of doxycycline and metronidazole to biofilm bacteria increased, but the MIC to planktonic bacteria of the same cell density increased to almost the same extent. In contrast to metronidazole, the MBC of doxycycline to biofilm cells increased considerably more than to planktonic cells. Supported by the Danish Medical Research Council.

2851 Community structure and enzyme activity in microcosm dental plaques.

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The objective was to examine the effects of different amino acid sources on community structure and on enzyme activity in *in vitro* microcosm dental plaques. Microcosm plaque biofilms were cultured in a multi-plaque artificial mouth from a plaque-enriched salivary inoculum under continuous supply of a saliva-analogue solution that contained protein in four different hydrolysis states: 0.25% mucin alone (unhydrolyzed), 5 g/L casein (unhydrolyzed) plus 0.25% mucin, 5 g/L of a partial digest of casein (Trypticase Peptone) plus 0.25% mucin, and a mixture of amino acids equivalent to 5 g/L of casein plus 0.25% mucin. Growth rates as measured by increase in wet weight over the 21 d experiment were variable but still within the range of growth rates *in vivo*. Analysis of endpoint community structure using phospholipid fatty acids showed that streptococci/actinomycetes predominated (≥ 50% of the fatty-acid suite) when unhydrolyzed protein was supplied, but comprised < 25% when hydrolyzed protein was present. These former communities also had fewer lipid-based generic groups (4) than did the latter (5 in the peptone-supplied plaque; 6 in the amino-acid-supplied plaque). Capnocytophaga occurred only in the amino-acid-supplied plaque, and Bacteroides was found in higher amounts in plaques supplied with peptone or with amino acids than in those supplied with unhydrolyzed protein: a more Gram negative and complex microflora occurred when simpler substrates were offered. Analysis of endpoint whole-plaque enzyme activities using API-ZYM™ plates showed that, in plaques grown on mucin alone, slightly higher levels of cysteine arylamidase and C16 lipase were present, but lower levels (2- to 4-fold) of 15 other enzyme activities occurred: complex substrates seemed to decrease enzymatic (metabolic) diversity. We conclude that community structure and whole-plaque enzyme activities are strongly dependent on environmental factors such as the degree of hydrolyzation of protein. Species composition of these plaques reflects those factors and mimics the composition seen in *in vivo* microenvironments.

2852 Topographical vitality pattern in dental plaque biofilms

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A vital fluorescence technique was combined with optical planes analysis using a confocal laser scanning microscope (CLSM). By combining these techniques the three-dimensional vitality distribution in plaque biofilms of a light (LPF) and a heavy plaque former (HPF) was evaluated. Both volunteers wore enamel chips in a splint for five days. After vital staining with fluoreseceine diacetate and ethidium bromide the specimens were processed for the CLSM examination. One µm thick optical planes were analyzed in the z-axis of these dental plaque biofilms. The plaque biofilm of the LPF was 16 µm high, sparse and showed low vitality, *i.e.*< 10%. On the other hand, that of the HPF was heigher (26 µm) and more vital, *i.e.* up to 30 %. In both instances the bacterial vitality increased from the enamel surface to the external part of the dental plaque. However, these overall data do not explain the spatial distribution of the vitality. Looking closely, it was found that voids in the plaque biofilm structure were outlined by layers of vital bacteria, while these were packed into layers of dead material. More CLSM-data of undisturbed plaque biofilms are therefore needed to reveal the complex three-dimensional adhesion and growth pattern of human dental plaque.

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2853 Artificial Bacterial Biofilms and the Confocal Laser Scanning Microscopic Analysis.

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Bacterial biofilms may be formed at various sites, including on mucos membrane, teeth and even in infectious lesions. To elucidate the structure and the function of biofilms, mucoid-type *Pseudomonas aeruginosa* organisms (strain PT1252) were centrifuged onto surface of a cover-glass and cultured in broth media supplied continuously (45 ml/hr). The biofilm structure of 4, 6, 8, 12 and 24 hr-old was visualized by fluorescent staining (SYTO9, PI and/or FITC-ConA). It was clearly demonstrated that No. of bacteria (10^4-10^9) can be estimated by the fluorescent intensity. Counting of each biofilm layer (1μm depth) revealed clearly that the biofilm developed 3-dimensionally to make up 9 layers after 12 hrs. The living and dead organisms were differentiated by SYTO9 and PI, respectively, *in situ* in biofilm, and about 10% organisms were dead in 12 hr-old biofilm. When biofilm of 12 hr-old were exposed to ciprofloxacin of minimum bactericidal concentration 6.26μ g/ml for 90 min, all the organisms were killed, but 11±1.3% (n=3) of organisms in 24 hr-old biofilm were still alive after the exposure for 120 min. These results indicate that the confocal laser scanning microscopical analysis of artificial biofilms was useful to elucidate bacterial functions in biofilms, and may lead to new quantitative estimation of bactericidal efficacy of antibacterial drugs.

2854 Detection of pH Gradients in Biofilms Using 2-Photon Excitation Microscopy.

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Gradients in key environmental parameters develop in biofilms. Defined, mixed culture biofilms of oral bacteria have been generated in a chemostat model (*J Appl Bacteriol* 80, 124-130, 1996). These biofilms were reproducible, but only 5-20 µm in depth, whereas plaque associated with fissures, or at the gingival crevice, is far deeper. This study aimed to produce biofilms of between 100-400 µm depth, and to examine these biofilms using a 2-photon excitation fluorescence lifetime microscope; pH gracients within the biofilms were measured directly using a pH-sensitive dye. A constant depth film fermenter (CDFF) was inoculated for 8 hours at 50 ml.h^{-1} with mixed cultures of up to 10 species of oral bacteria grown in a chemostat; fresh growth medium was supplied to the biofilms in the CDFF, for 10 days, and the biofilms were then removed. The microbial composition of the biofilms was determined using selective and non-selective agar media. Replicate biofilms were overlaid with 2% (w/v) sucrose for 1 hour, and then carboxyfluorescein added. The distribution of pH was then determined by examining fluorescence lifetime images throughout the depth of the biofilm. Viable counts showed that diverse mixed culture biofilms developed, including aerobic, facultative and anaerobic species. The 2-photon excitation microscope was able to distinguish individual bacteria to a depth of up to 140 µm. The sucrose/carboxyfluorescein images indicated zones with pH as low as pH 3.0, in some cases adjacent to areas with much higher pH (pH > 5.0). The CDFF apparatus can generate deep biofilms to simulate plaque from at-risk sites. The 2-photon excitation microscope has been used successfully to resolve images as deep as 140 µm, and allowed the real-time imaging of pH in biofilms.

2855 Effect of endogenous proteins on *Candida* biofilm formation on acrylic

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It is known that fibronectin(FN), mannan-binding proteins(MBP), mucin and concanavalin-A(Con-A) binding material may be involved in candidal biofilm formation on acrylic surfaces (Nikawa et al. Microbial Ecol Health Dis 9: 35-48, 1996). These proteianceous substances are derived in vivo from endogenous sources such saliva, serum and other microbial products. In the current study we further examined, using the method of Nikawa et al (1996) the effect of salivary or serum proteins, such as mucin, fibronectin and MBP, on in vitro candidal biofilm formation. A single oral isolate each of *Candida albicans* , *C. tropicalis* and *C. glabrata* were used in the study. The biofilm activity on acrylic strips laced with the appropriate proteins and the yeasts was determined using the previously described adenosine triphosphate (ATP) assay. The biofilm activity was assessed after supplementation of human saliva/serum with various proteins. Supplementation of human mixed saliva with FN had no significant effect on biofilm activity whereas supplementation of human serum with FN increased the biofilm activity of *C. glabrata* as compared with the controls. In contrast, the activity of the biofilm which developed on either the mucin-coated or FN-coated acrylic strips was significantly less than that of the controls (ANOVA, p<0.01), suggesting that salivary mucin and FN in isolation may not support the biofilm formation of *Candida*. Pretreatment of serum with antiFN monoclonal antibody significantly reduced biofilm activity of all *Candida* isolates (p<0.01). This observation implies that FN together with other serum components such as fibrinogen may modulate *Candida* biofilm formation. Similarly, pretreatment of serum with antiMBP monoclonal antibody or Con-A tended to significantly reduce the biofilm activity of *C. albicans/tropicalis* (p<0.05). Thus it seems that the cell surface mannnan in combination with MBP in serum play an equally important role in *Candida* biofilm formation. To conclude our results tend to suggest that a number of endogenous proteins present both in saliva and serum contribute to *Candida* biofilm formation in a very complex manner.

2856 Factors involved in *Candida* biofilm formation on acrylic surfaces

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Despite the realization that successful colonization of denture surface is an important step in the denture stomatitis, the role played by denture pellicle during the colonization process and subsequent biofilm formation is poorly understood. In addition, it is likely that during biofilm formation, cell surface properties of *Candida*, may be involved and thus a deeper understanding *Candida* biofilm formation necessitates clarification of such intertwined relationships. The purpose of the present study was to analyze fungal properties, such as acid production, cell surface hydrophobicity, germ tube formation and thigmotropism, involved in the *Candida* biofilm formation on saliva, serum, and saliva-serum coated acrylic surfaces using *C. albicans* (4 isolates), *C. glabrata* (3) and *C. tropicalis* (3). Biofilm assay was carried out according to our previous study (Microbial Ecol Health Dis 9: 35-48, 1996) and the biofilm activity determined by ATP (adenosine triphosphate) analysis. The biofilm activity varied depending upon both the isolate and the pellicle. As compared with the uncoated control, significantly increased biofilm activity on the pellicle (particularly serum-coated) strips, was observed with three isolates of *C. albicans* and another of *C. glabrata*. However, the biofilm activity on saliva-coated strips was significantly lower than that of the protein-free control strips, with all isolates of *C. tropicalis* and one isolate of *C. glabrata*. When the biofilm activity and candidal attributes were explored, a significant positive correlation was observed between the biofilm activity and rate of acid production (r=0.728, p<0.05) and thigmotropism (r=0.823, p<0.01), but not cell surface hydrophobicity (r=0.539, p>0.05). Taken together our in vitro data imply that candidal biofilm formation on acrylic surfaces is a complex phenomenon regulated by a multiplicity of factors operating intra-orally.