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Antibiotic resistance in an *in vitro* subgingival biofilm model

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Introduction: The purpose of this study was to utilize an *in vitro* biofilm model of subgingival plaque to investigate resistances in subgingival biofilm communities to antibiotics commonly used as adjuncts to periodontal therapy.
Methods: Biofilms were grown on saliva-coated hydroxyapatite supports in trypticase-soy broth for 4 h–10 days and then exposed for 48 h to either increasing twofold concentrations of tetracycline, amoxicillin, clindamycin, and erythromycin or therapeutically achievable concentrations of tetracycline, doxycycline, minocycline, amoxicillin, metronidazole, amoxicillin/clavulanate, and amoxicillin/metronidazole.
Results: Concentrations necessary to inhibit bacterial strains in steady-state biofilms were up to 250 times greater than the concentrations needed to inhibit the same strains grown planktonically. In the presence of therapeutically available antibiotic concentrations, significantly higher proportions of the biofilms remained viable as the biofilms reached steady-state growth. The combinations of amoxicillin/clavulanate and amoxicillin/metronidazole were the most effective in suppressing growth. These combinations were particularly effective against biofilms up to and including 7 days of age and inhibited 90% or more of the bacteria present relative to untreated controls. As the biofilms approached steady state, these combinations were less effective with 50–60% of the bacteria retaining viability.
Conclusion: Most, but not all, species of subgingival bacteria are considerably more resistant in biofilms than in planktonic cultures. Resistance appeared to be age-related because biofilms demonstrated progressive antibiotic resistance as they matured with maximum resistance coinciding with the steady-state phase of biofilm growth.

Key words: antibiotics; biofilm; planktonic; resistances; subgingival

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Periodontitis has replaced caries as the primary cause of tooth loss in the USA and other industrialized countries. In addition, strong associations have been implicated between periodontitis and a number of other disorders such as low birth weight (5), heart disease (12), and stroke (17). Although a number of factors are involved, periodontitis results from a multi-species bacterial infection and the host's response to this infection. The disease is usually successfully controlled by mechanical debridement, but some cases benefit from adjunctive antibiotic therapy (43, 44). In the latter, the question is which antibiotic will be the most effective. The subgingival plaque, in both health and disease, exists as a complex biofilm consisting of a multi-species consortium that may consist of various combinations of any of the hundreds of bacterial species that are postulated to colonize the surfaces of the oral cavity. Out of this multitude of different species, several specific species have been found to be closely associated with disease initiation and progression and have been targeted for elimination from the subgingival plaque (35–37). The main focus of periodontal treatment is the thorough removal of plaque, calculus and plaque products, often by nonsurgical scaling and root planing. However, such therapy may not eliminate all periodontal pathogens from the subgingival environment. Deep periodontal pockets with grooves, furcations and concavities, adhesion to soft oral tissues, and tissue invasion provide periodontopathic bacteria with reservoirs for future re-colonization of the periodontal pocket. In such instances, mechanical debridement may be combined with adjunctive antimicrobial therapy.

In the past, the levels of an antibiotic obtained in the gingival crevicular fluid

were compared with the antibiotic concentrations necessary to inhibit or kill specific bacterial species associated with diseased periodontal sites. Such information was used to provide some guidance as to which antibiotics might be the most effective as adjuncts to periodontal therapy. However, the antibiotic concentrations required to inhibit or kill periodontal bacteria were determined in planktonically grown bacteria and not in bacteria within a complex biofilm where antibiotic resistance is postulated to be several magnitudes higher (34, 44).

The purpose of this study was twofold. A previous *in vitro* biofilm model of subgingival plaque (46) was used to investigate differences in the antibiotic concentrations required to inhibit subgingival bacterial strains in biofilm mode of growth relative to those same strains grown planktonically. This was investigated by exposing biofilms to increasing concentrations of antibiotics and then determining the surviving species present. These species were then grown planktonically to obtain a direct comparison of the susceptibilities and resistances under planktonic and biofilm growth modes. The second objective was to determine the effect of therapeutic levels of several antibiotics, commonly used adjunctively, on biofilms at different stages of development to determine if the maturity of the biofilm influenced resistance to antibiotics.

Materials and methods

Sampling

After obtaining informed consent, microbial samples of subgingival plaque were collected from five individuals with no evidence of periodontal disease and from 10 patients with nonaggressive adult periodontitis. The criteria used for selection of the latter included: subjects between the ages of 35 and 65 years with no known systemic diseases, who had not received antibiotics within the previous 3 months, and who were not taking medications that might influence the subgingival flora. The sites samples had pocket probing depth ≥5 mm and clinical attachment loss ≥3 mm. Bleeding on probing with and without suppuration was sometimes present. Samples were collected by inserting a sterile absorbent paper point (Henry Schein, Melville, NY) to the depth of the sulcus and moving it laterally along the surface of the tooth and the sulcular epithelial lining. The paper point sample was immediately placed into 1-ml Amies transport medium (3) supplemented with 0.5% gelatin (Fisher Scientific, Ocala, FL) and reduced with 0.1% sodium thioglycollate (Fisher) and stored overnight at 4°C.

Saliva collection and processing

Unstimulated saliva was obtained in 5-ml samples from the same subjects who had donated subgingival plaque as previously described (46). In brief, each saliva sample was diluted (1 : 10) with prereduced, anaerobically-sterilized Ringer solution (21), containing 0.05% cysteine (Sigma Chemical Co, St Louis, MO) as a reducing agent, centrifuged, at $2{,}000 \times g$ for 10 min, to remove any particulate matter, and the supernatant was filter sterilized.

Biofilm development

Sterile ceramic calcium hydroxyapatite discs, 5-mm diameter and 2-mm thickness, (Clarkson Chromatography Products, Williamsport, PA) were coated with 10% sterile saliva overnight at room temperature and placed in the wells of a six- or 12-well tissue culture plate containing either 2 ml or 4 ml trypticase-soy broth (TSB) (BBL®; Becton Dickinson, Sparks, MD) respectively. Each well was inoculated with 50 μl sonically dispersed subgingival plaque. The discs were incubated in an anaerobic chamber (10% H_2, 5% CO_2, and balance N_2) at 37°C for up to 10 days with a change to fresh medium at 48-h intervals. Biofilm-containing discs were cultivated for 4, 12, 24, and 48 h, and for 5, 6, 7, and 10 days. In this model, steady-state or climax biofilms are considered to require 10 days of growth (46). Biofilms exposed to amoxicillin and to amoxicillin/clavulanate were also exposed at 8 and 9 days as well as at the previous times.

Biofilm vs. planktonic susceptibilities

The susceptibilities of the biofilms and of planktonically grown bacterial strains were determined for tetracycline, amoxicillin, erythromycin and clindamycin. Susceptibilities of the planktonically grown strains recovered from the biofilms were only determined to tetracycline, amoxicillin and clindamycin because erythromycin showed very little effect on the biofilms. Subgingival plaque samples from five periodontally healthy individuals and from five subjects with adult nonaggressive periodontitis were used to initiate biofilms. Each biofilm was grown for 10 days (climax stage) and then exposed for 48 h to the selected antibiotic at twofold increasing concentrations ranging from 8 μg/ml to 2048 μg/ml (three separate determinations for each concentration per biofilm). After exposure to each antibiotic concentration, the surviving bacteria, were recovered, serially diluted, plated on trypticase-soy agar supplement with 5% defibrinated sheep blood, 0.005% hemin and 0.0005% menadione (TSBA-HK), and incubated anaerobically for 5–7 days. Each major colony type present was subcultured and identified. Inhibition in the biofilm was considered to be the lowest antibiotic concentration that inhibited the growth of a particular strain. These strains, from the next lowest concentration that permitted their growth in the biofilm, were grown planktonically in peptone–yeast extract–glucose broth (21) for susceptibility testing as previously described (45). The antibiotic concentration required to inhibit the particular bacterial strain in the biofilm was compared to the concentration required to inhibit the same strain grown planktonically.

Effect of biofilm maturity on susceptibilities

Biofilms from 4 h to 10 days old were exposed to clinically achievable gingival fluid levels of each of the following: 2 μg/ml tetracycline–HCl (Sigma) (16), 4 μg/ml minocycline–HCl (Sigma) (15), 4 μg/ml doxycycline hylate (Sigma) (28), 4 μg/ml amoxicillin (Sigma), amoxicillin/clavulanate, 4 and 2 μg/ml respectively, (RPI Corp., Mount Prospect, IL) (41), 2 μg/ml clindamycin–HCl (Sigma) (42), 16 μg/ml metronidazole (Sigma) (30), and a combination of 4 μg/ml amoxicillin and 16 μg/ml metronidazole. All stock solutions except metronidazole were made by dissolving the desired antibiotic concentration in 20 ml de-ionized water and filter sterilizing the solution. Metronidazole was dissolved in 1 ml dimethylsulfoxide, brought to a final volume of 20 ml in de-ionized water, and filter-sterilized. To obtain the concentration of antibiotic desired, the stock solutions were serially diluted (twofold) in sterile de-ionized water. Each biofilm was transferred from TSB to a single well of a 12-well tissue culture plate containing 1 ml double-strength TSB. A 1-ml aliquot of the antibiotic dilution at twice the desired final concentration was then added. Controls without antibiotics were prepared by adding 1 ml of sterile de-ionized water to 1 ml of double-strength TSB. The biofilms were exposed to the antibiotic concentration for 48 h, dispersed, serially diluted and plated

on TSBA-HK to determine the number of viable colony-forming units (CFUs) present relative to paired untreated controls.

Bacterial identifications

Bacterial strains and subcultures were identified to genus and species, or bacterial taxa if a species name had not been assigned, based on the quantity and quality of the bacterial cellular fatty acids measured by capillary gas–liquid chromatography as described by Moore (26), using a capillary gas–liquid chromatograph (Model 5890: Hewlett Packard, Avondale, PA) equipped with HP autosampler and connected to a Dell computer with MIDI software (Microbial ID, Newark, DE) for instrument control and analysis. Computer-assisted identifications were based on the MIDI Anaerobic Bacteria Library (MOORE5, Microbial ID).

Statistical methods

Differences in CFUs obtained were tested using nonparametric statistics to avoid the influence that high counts may have on the mean. The Wilcox signed rank was used to detect differences between paired observations; the Mann–Whitney test was used to detect differences between two unpaired sets of observations. The Kruskal–Wallis test was used to test the hypothesis that two or more groups all come from the same distribution as opposed to the alternative that at least one comes from a different observation. Differences in per cent inhibition by antibiotics relative to untreated controls were tested using parametric distributions because a normal distribution was assumed. For this, a one-way analysis of variance was used to detect if differences existed and a paired or unpaired *t*-test was used to detect where differences lay. In all instances, a minimum of three determinations for each biofilm was used.

Results

Effect of antibiotics on biofilm grown bacteria

The effects of tetracycline, amoxicillin, clindamycin and erythromycin were independently determined on biofilms grown to the climax stage (10 days old, $\sim 10^9$ CFUs) by exposing climax-grown biofilms to a single antibiotic concentration (three determinations each) for a 48-h period and determining the CFUs and the species that survived. The major surviving colony types were subcultured, identified, grown planktonically, and their susceptibilities to the antibiotic were determined. The effect of increasing the dilutions for each antibiotic twofold on the total viable counts is given in Fig. 1. Tetracycline was the most effective antibiotic in suppressing the total viable counts, followed by amoxicillin. Tetracycline gave roughly a 4 log reduction in the number of viable counts at concentrations of 512 μg/ml and higher. This was equivalent to killing ~99.99% of the cells present; however, this still left 10^5 viable cells. The survivors at the higher concentrations for both tetracycline and amoxicillin consisted primarily of streptococci, *Actinomyces* and *Veillonella* species. Both clindamycin–HCl and erythromycin were relatively ineffective against the climax biofilms, regardless of the concentration tested, and gave, at best, a 1-log decrease.

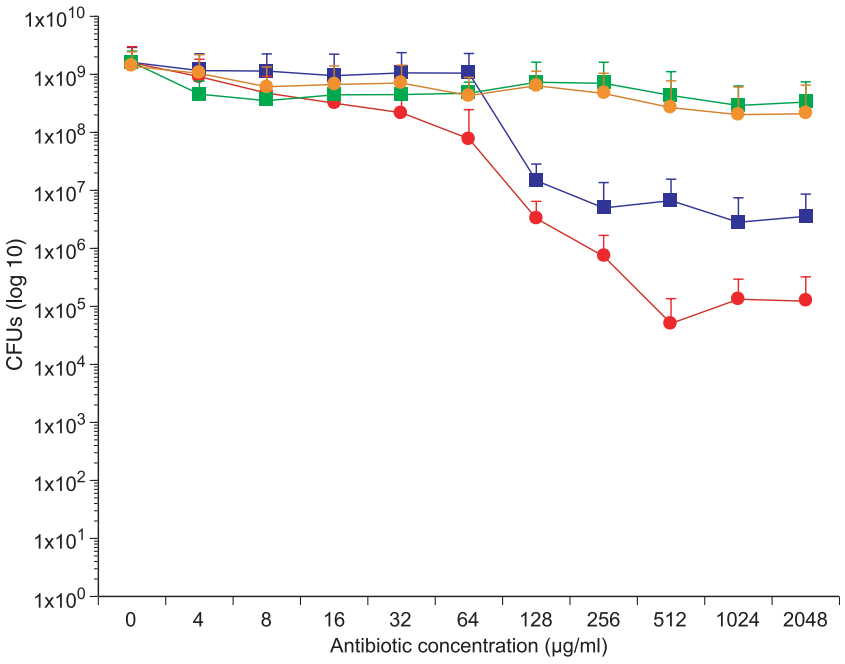

Fig. 1. Effect of increasing antibiotic concentrations on steady-state biofilms (10 days old) expressed as the CFUs (mean ± SD) recovered after 48 h of exposure to each antibiotic concentration. Antibiotics tested: tetracycline (●), amoxicillin (■), clindamycin (●), erythromycin (■).

Comparison of antibiotic effect on the same bacteria grown in biofilm and planktonic cultures

Table 1 presents the antibiotic concentrations for tetracycline-HCl, amoxicillin and clindamycin-HCl that were required to inhibit the predominant bacterial species recovered from the biofilms described above and for these same strains grown planktonically. Statistically, the antibiotic concentrations required to achieve inhibition in the biofilms were significantly higher than those required to inhibit the same strains grown planktonically ($P < 0.0001$).

Effect of therapeutic concentrations of antibiotics on viable biofilm mass

To examine the effects of antibiotics at concentrations equivalent to therapeutic levels, biofilms from five subgingivally healthy subjects and five subjects with periodontitis were grown in triplicate. Biofilms ranging in age from 4 h to 10 days were exposed to systemically achievable concentrations of tetracycline, doxycycline, minocycline, amoxicillin, metronidazole, amoxicillin/clavulanate, and a combination of amoxicillin and metronidazole. As no statistically significant differences were found in the antibiotic effects on biofilms derived from healthy sites relative to diseased sites with the exception of metronidazole, the data were combined. Figure 2 gives the mean per cent survival of the viable biofilm mass, relative to untreated controls, for each antibiotic at each time-point tested. Statistical analysis (unpaired *t*-test) revealed significant reductions ($P < 0.01$) in biofilms exposed to tetracycline at every growth point when compared with untreated controls. Although the differences were not statistically significant, both doxycycline and

Table 1. Concentrations (μg/ml) of three antibiotics required to inhibit bacterial species grown both in biofilms and planktonically

	Tetracycline		Amoxicillin		Clindamycin	
Bacterial taxa[1]	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic
Actinomyces naeslundii	512–2048	32–64	1024	≤ 8	512	128
A. meyeri	64	≤8	512	≤8	512	64
A. georgiae	128	512	128	≤8	32	16
Bacteroides species	128–512	128	128–512	128	128	≤8
Bifidiobacterium species	32	16	32	16	32	16
Campylobacter species	>2048	64	256	≤ 8	1028	≤ 8
Eubacterium species	128	128	ND	ND	512	128
Fusobacterium alocis	256	≤8	16	≤8	128	≤8
F. nucleatum	256	≤8	128	≤8	64	≤8
F. prausnitzii	>2048	≤8–512	1024	≤8–32	1024	≤8
F. russi	1024	≤8	512	≤8	512	≤8
Prevotella intermedia/nigrescens	1024–2048	≤8–32	1024	≤8–128	128	≤8
P. loescheii	1024	64	512	≤8–32	128	≤8
Streptococcus intermedius	1024	32	2048	16–256	1024	32
S. parasanguis	>2048	32	2048	≤8	>2048	64
S. sanguis	512–2048	16–128	1024	32	>2048	32
Veillonella atypica	128–2048	≤8–16	>2048	≤8	1024	≤8
V. dispar	>2048	≤8–512	1028	≤8	1024	≤8
V. parvula	>2048	≤8	>2048	≤8	>2048	≤8

[1]Identified using the VPI Anaerobe database (Moore 6, Microbial ID, Inc.).

minocycline resulted in slightly fewer survivors than did tetracycline. However, as the biofilms matured, the percentage of viable cells surviving exposure to the tetracyclines increased in number. In biofilms exposed to amoxicillin, significant reductions ($P < 0.01$) were observed at all time-points through 7 days. Exposure to metronidazole resulted in significant reductions ($P < 0.01$) in periodontitis-derived biofilms at all time-points and in healthy biofilms at all growth-points with the exception of days 7 and 10. Despite this reduction in cell numbers, the percentage of viable cells surviving ranged from 10–30% in young biofilms (4 h to 5 days) to 60–95% in older biofilms (7–10 days). All biofilms exposed to the combination of amoxicillin and metronidazole and to amoxicillin/clavulanate were found to have a significant reduction in viable cell numbers ($P < 0.01$) at all growth points tested.

Since a statistically significant increase was observed with both amoxicillin-treated and amoxicillin/clavulanate treated biofilms from day 7 to day 10, the experiment was repeated and included biofilms grown for 8 and 9 days as well as the previous times. Roughly 30% of the cells were resistant to amoxicillin at 8 days compared with 14% at 7 days (Fig. 3). There were no appreciable differences between 7- and 8-day-old biofilms when exposed to amoxicillin/clavulanate. However, a significantly greater number of cells were resistant to both amoxicillin and amoxicillin/clavulanate at day 9 and were comparable with the number of cells resistant at day 10.

Discussion

The phenomenon of increased antimicrobial resistances and reduced susceptibilities in biofilms is well recognized. Therefore, it is rather surprising that very few investigations have sought to document the magnitude of the difference in antibiotic susceptibilities between biofilm and planktonically grown bacteria. In the few that have, documentation has almost exclusively been limited to mono-species biofilms relative to broth cultures of *Staphylococcus aureus* (8, 22, 27) and *Pseudomonas aeruginosa* (1, 13, 20). In the oral cavity the supra- and subgingival plaque consists of a consortium of multiple species in biofilms. Several biofilm models have been described that could be or have been applied to the study of multi-species oral biofilms and the effects of various antimicrobials (2, 14, 18, 51). However, none of these have compared the antibiotic effect on biofilms relative to the constituent species recovered from the biofilms and grown planktonically.

In this study, the effects of increasing twofold concentrations were independently determined for four antibiotics against climax biofilms initiated with subgingival plaque. The biofilms were directly inoculated with the plaque sample, grown for 10 days to climax stage in TSB, exposed for 48 h to increasing twofold antibiotic concentrations, the survivors at each concentration were isolated, subcultured once, and grown in TSB broth. Thus, the effects of antibiotics on planktonic cultures were effects on wild strains that had not been serially passed in the laboratory. No appreciable microbial effects were observed, as determined by viable CFUs, for either erythromycin or clindamycin even at concentrations as high as 2 mg/ml (2000 μg/ml). The net reduction in viable CFUs recovered was less than 1 log10. Amoxicillin gave a reduction of 2–2.5 logs at concentrations of ≥128 μg/ml. This was equivalent to killing 99% of bacteria. Of the four antibiotics, tetracycline was the most effective and appeared to be more dose dependent because the total CFUs recovered were progressively less with each increase in concentration from 16 to 512 μg/ml. However, concentrations greater than 512 μg/ml provided no additional effect. The bacterial species recovered after exposure to each increasing concentration consisted of both Gram-positive and Gram-negative bacteria. The Gram-negative *Prevotella* and *Veillonella* species as well as *Fusobacterium prausnitzii* were all relatively resistant to tetracycline and amoxicillin at concentrations ≥1024 μg/ml.

It has been postulated that the antibiotic concentrations required to inhibit or kill bacteria in biofilms may be from 500-fold to 1000-fold greater than those required to inhibit or kill planktonically grown strains. Our data clearly demonstrate that a significantly higher amount of drug is required to have an inhibitory effect in the biofilm than it in planktonically grown bacteria of the same strain. However, the susceptibilities for some biofilm-grown species, such as *Bifidiobacterium* and *Eubacterium*, were not appreciably different when grown planktonically. Other species, however, required at least a 250-fold increase in

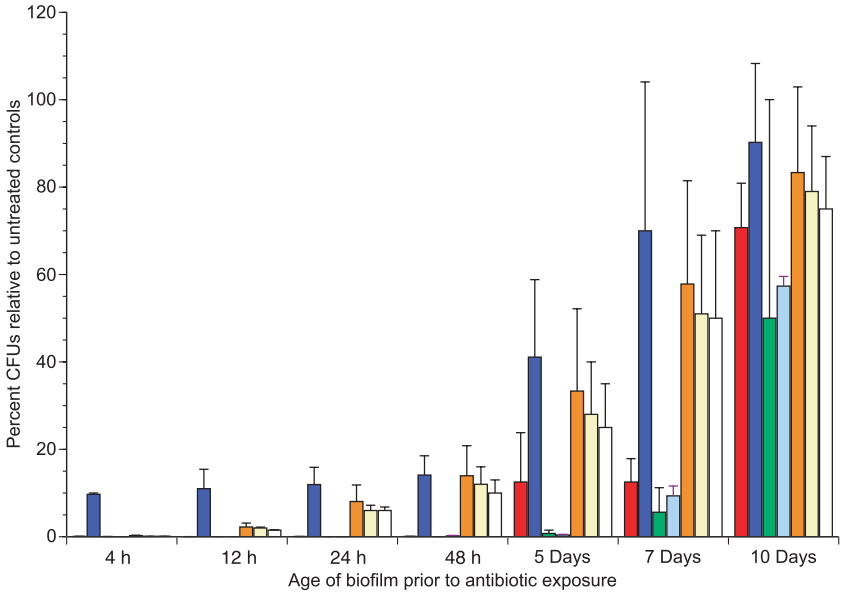

Fig. 2. Effect of therapeutic antibiotic levels on biofilm CFUs (means ± SD) at various time-points leading up to and including steady-state. Antibiotics tested: amoxicillin (■), metronidazole (■), amoxicillin/metronidazole (■), amoxicillin/clavulanate (■), tetracycline (■), doxycycline (■), minocycline (□).

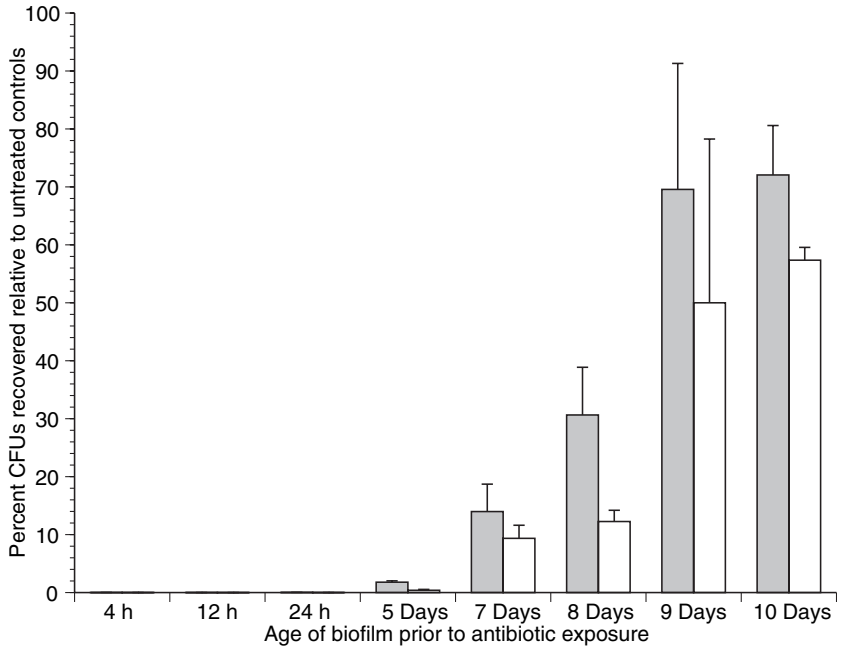

Fig. 3. Effect of therapeutic levels of amoxicillin and amoxicillin/clavulanate on biofilm CFUs (means ± SD) from 4 h to 10 days. Amoxicillin (■), amoxicillin/clavulanate (□).

antibiotic in the biofilm mode compared with when they were grown in broth. This could easily be two- to fourfold higher because the lowest concentration tested was 8 μg/ml while concentrations as low as 0.25 μg/ml are often used in susceptibility testing of broth cultures. In some species, e.g. *Actinomyces meyeri*, *Actinomyces georgiae*, *Filifactor alocis*, a considerable increase in antibiotic concentration was required to inhibit growth in the biofilm mode for some antibiotics but not for others. These results are in agreement with those of Ceri et al. (9) who reported that mono-culture biofilms of *Escherichia coli*, *P. aeruginosa*, and *S. aureus* required 100-fold to 1000-fold higher antibiotic concentrations for certain antibiotics to be effective in biofilms relative to the same strains grown planktonically, while for other antibiotics the inhibitory concentrations were similar.

Several mechanisms have been proposed to account for the differences in antibiotic susceptibilities in biofilms relative to planktonically grown cells. These have included oxygen limitation (6), antibiotic penetration into the biofilm (4), and the presence of a small subpopulation of 'persister' cells (23), as well as other possible mechanisms. Interestingly, Boriello et al. found that young biofilms formed with a mono-culture of *P. aeruginosa* were not protected by oxygen deprivation to the same extent as more mature biofilms (6). We thought that the age or maturity of the biofilm might also be an important contributor to increased resistance. To test this, biofilms were grown for a specific period ranging from very young to climax stages and then exposed to therapeutically achievable levels of an antibiotic for 48 h. The viable counts obtained were compared to paired controls of the same age that had not been exposed to the antibiotic. Exposure to metronidazole alone gave the least suppression of viable counts relative to the other antibiotics tested. This was not surprising considering that metronidazole specifically targets anaerobic bacteria and has little or no effect on aerobes or facultative organisms. However, it does not explain the steady increase in the proportions that were resistant as the biofilms aged. Previous studies have indicated that it is the gram-negative anaerobes that increase most in aging biofilms in this model (46). Resistance to the three tetracycline antibiotics demonstrated a gradual increase for the first 48 h but then showed a more rapid increase from 5 to 10 days. Although, slightly less resistance was observed at each time-point for doxycycline and minocycline relative to tetracycline, the differences were not statistically significant ($P = 0.88$). At therapeutically obtainable levels, amoxicillin and the amoxicillin combinations (amoxicillin/clavulanate and amoxicillin/metronidazole) were the most effective. However, there was a significant increase ($P < 0.01$) in the proportion that were resistant from day 7 to day 10. To determine where this difference occurred, the experiment was repeated for amoxicillin and amoxicillin/clavulanate with samples taken at days 8 and 9 as well as at the other time points. Although an increase in the resistant proportion was observed for amoxicillin at day 8, there was little change for amoxicillin/clavulanate. The major increase in the resistance to both occurred on the 9th day. It is also interesting that the

most variation, expressed as standard deviation, in the percentages that grew in the present of these two antibiotics was the greatest on day 9. The reductions in biofilm mass caused by exposure to amoxicillin/clavulanate were significantly ($P < 0.01$) greater than those obtained by the same concentration of amoxicillin during all stages of growth except at day 10 ($P > 0.10$). A suppression rate of 85% or more was observed from 4 h to 8 days. However, at 9 days, roughly 50% of the bacteria in the biofilm survived. We did not determine the β-lactamase activities present in this study. We have reported that β-lactamase production in planktonically grown cultures occurs in late log phase and/or early stationary growth phase (47). The same could be true in the biofilm. However, β-lactamase cannot account for all of the viable counts obtained at days 9 and 10 because 50–60% of biofilm counts grew in the presence of amoxicillin/clavulanate. These data could be interpreted to indicate that adjunctive antibiotic therapy is most effective against young developing biofilms. This coincides with clinical findings that adjunctive antibiotic therapy is best performed immediately following a thorough mechanical debridement (19, 32, 33, 43, 44, 49, 50).

The increase in resistance to the antibiotics tested was considerably greater than could be accounted for by just the increase in viable bacterial mass. Various mechanisms to account for the increased resistances to antimicrobials in biofilms have been postulated. Several of these mechanisms seem to occur in conjunction with the final stages of biofilm maturation (29). Reduced penetration into the biofilm may result in antibiotic inactivation because of secretion of certain enzymes, such as β-lactamases, or binding of the agent by the exopolysaccharide matrix. The exopolysaccharide could inhibit antimicrobial penetration by either binding the antimicrobial (10, 40) or serving as a protective coating that prevents or delays diffusion through the biofilm (38, 39). The complex heterogeneity within biofilms is evidenced by studies analyzing different microenvironments throughout the biofilm that differ in metabolic activity (48), pH , and oxygen distribution (11). The 'biofilm phenotype' is a collective term used to describe a biologically programmed response to growth on a surface that involves specific physiologies and patterns of protein and gene expression that are quite different from those of planktonic cells (24, 31) and have been linked to aspects of antimicrobial resistance (7, 24, 25). Increased resistance to antimicrobials is likely a combination of all of these mechanisms and may involve many, if not all, of these factors working together in unison as the biofilm matures.

We think the *in vitro* model utilized in this study mimics what occurs when the subgingival flora is challenged, *in vivo*, by antimicrobial agents and may be of use in elucidating mechanisms of antibiotic resistance in the subgingival biofilm.

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