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Susceptibilities of *Actinobacillus actinomycetemcomitans* biofilms to oral antiseptics

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The susceptibilities of *Actinobacillus actinomycetemcomitans* cultures, grown as 1- or 3-day-old biofilms or as planktonic suspensions, to concentrations of chlorhexidine digluconate, cetylpyridinium chloride or triclosan used in commercial mouthwashes were compared. Three-day biofilms were the most resistant form of the organism and chlorhexidine was the most active antiseptic. Comparison of solutions of the pure antibacterial agent with commercial products containing the same concentration of antiseptic showed little difference in in-vitro activities. The results emphasise that the testing of antimicrobial mouthwashes should be performed on bacteria grown as biofilms.

Introduction

Actinobacillus actinomycetemcomitans is one of the main causative organisms of chronic inflammatory periodontal disease [1]. It is a gram-negative capnophilic rod with a natural habitat at the margin of the tooth and gum. It is especially associated with localised juvenile and refractory periodontitis, and its presence, together with the immune factors it triggers, are useful diagnostic markers of periodontal disease [2]. Involvement of *A. actinomycetemcomitans* in the aetiology of periodontitis is indicated by its regular isolation from diseased lesions, the fact that inoculation of gnotobiotic rats induces similar disease states, and that eradication of the organism results in successful therapy [3].

The virulence of *A. actinomycetemcomitans* results from several factors. It maintains itself within the gingival crevice by adhering to the tooth pellicle, to epithelial cells or to other bacteria. Host tissue is then destroyed by exosecretions such as epitheliotoxin, leucotoxin, bone resorbing factors, collagenase or fibroblast-inhibiting factor [4]. Host antibodies are inactivated and initiation of the host inflammatory response by a variety of cytokine-stimulating components causes further cellular damage.

A. actinomycetemcomitans is found in biofilms (i.e., in dental plaque) on the tooth surface. These contain large numbers of slow-growing cells, which are inherently more resistant to antimicrobial agents than planktonic suspensions [5]. Traditional treatment of periodontal disease consists of the removal of subgingival plaque by mechanical procedures, such as root planing, to eradicate the disease-inducing organisms. Oral hygiene is considered pivotal in both prophylaxis and therapy. However, most individuals do not comply with recommended oral hygiene procedures, and there is currently great interest in supplementing these with antimicrobial agent-containing mouthwashes and toothpastes [6, 7]. These suppress gingival pathogens and reduce inflammation, but are limited in effect because of their short in-use contact time and the resistance of oral biofilms [8]. This has led to the re-formulation of mouthwash products in an attempt to increase adherence to the acquired tooth pellicle, thus prolonging contact time.

Little published information is available on the susceptibility of *A. actinomycetemcomitans* biofilms to solutions of oral antiseptics or to formulated mouthwashes. This study investigated the effects of three commonly used oral antiseptics on biofilms and planktonic suspensions of *A. actinomycetemcomitans*, and compared these with the activities of commercial products containing the same concentrations of active agent.

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Materials and methods

Production of biofilms

Biofilms of *A. actinomycetemcomitans* strain HG1174 were grown on cellulose nitrate membrane filters as described previously [9, 10]. Quantities (20 µl) of an overnight culture in Wilkins Chalgren (WC) broth (Unipath, Basingstoke, Hants) were spread on to 13.0-mm diameter cellulose nitrate membrane filters (0.22 µm pore diameter; Whatman Ltd, Maidstone, Kent) placed on the surfaces of Wilkins Chalgren agar plates. Six membranes were accommodated on each plate and were then incubated for either 24 or 72 h in an anaerobic cabinet (Don Whitley Ltd, Shipley, Yorks) at 37°C in an atmosphere of N₂ 80%, H₂ 10%, CO₂ 10%.

Experimental procedures

Each biofilm was carefully transferred from the agar to a vial containing either 10 ml of the appropriate test agent or a control diluent, and left in contact for 1, 5 or 60 min at 25°C. It was then transferred to 10 ml of Bacto D/E neutralising broth (Difco Ltd, East Molesey) and vortex mixed for 1 min to resuspend the organisms. The resulting suspension was diluted in WC broth and viable bacteria were counted on blood agar plates by the method of Miles and Misra [11]. Plates were incubated anaerobically for 3 days at 37°C. Control unexposed biofilms were treated similarly. Each data point represents the mean (with 95% confidence limits) of the cfu's detached from six biofilms. Samples of sterile diluent in which control untreated biofilms had been incubated were also transferred to neutralising broth, diluted and counted to check that cells were not lost from biofilms during incubation. The significance of the difference of two means was assessed by Student's *t* test.

Testing planktonic suspensions

Planktonic suspensions were grown anaerobically in 10 ml of WC broth for 24 h at 37°C; 1 ml was then added to 10 ml of antiseptic solution or control diluent at 25°C. After the required exposure time, 1-ml samples were transferred to 9 ml of neutralising broth, which was diluted, and the number of viable organisms was determined.

Oral antiseptics

The antiseptics used were chlorhexidine digluconate, cetylpyridinium chloride (both from Sigma), and triclosan (SmithKline Beecham Healthcare, Weybridge, Surrey). The first two were dissolved in saline, the third in ethanolic saline 5%. Commercial products were: Corsodyl (SmithKline Beecham) containing chlorhexidine digluconate 0.2%, preservatives and flavouring; Merocet (Marion Merrell Dow Ltd, Uxbridge, Middlesex) composed of cetylpyridinium chloride 0.05%,

alcohol, sorbitol, polyethylene glycol-40, sodium fluoride and sodium saccharin; Plax (Colgate-Palmolive Ltd, Guildford, Surrey), which contains triclosan 0.03%, a co-polymer of methoxyethylene and maleic acid 0.125% and sodium fluoride.

Results

Controls

Overnight planktonic cultures of *A. actinomycetemcomitans* gave viable counts of $(3.0 \pm 0.1) \times 10^6$ cfu/ml. These were diluted 1 in 11 into the antiseptic solutions or into control diluents. Disruption of 1- and 3-day biofilms gave total viable counts of $(3.7 \pm 0.2) \times 10^7$ and $(4.0 \pm 0.4) \times 10^7$ cfu respectively. After exposure, these were resuspended in 10 ml of neutralising broth before further dilution and plating. Controls designed to test the effect of diluents on viability showed no significant differences over 1-h exposure periods compared to counts at time zero. Controls also showed no significant loss of organisms from biofilms suspended in sterile diluent for up to 1 h.

Susceptibilities of 1- and 3-day-old biofilms and planktonic suspensions

Comparisons of the susceptibilities of biofilms and planktonic suspensions to concentrations of the oral antiseptics used in commercial mouthwashes are shown in Fig. 1. After exposure to chlorhexidine 0.2% for 1 min, the viable count of planktonic cells compared to controls was significantly reduced ($p < 0.001$), as were counts of the 1- and 3-day-old biofilms. However, exposure to both cetylpyridinium chloride 0.05% and triclosan 0.03% solution for 1 min was not significantly lethal to planktonic cells, or to 1- or 3-day-old biofilms.

The relative susceptibilities of planktonic cells and of biofilms to chlorhexidine became more apparent after exposure for 5 and 60 min (Fig. 1). At 5 min, the survival of 3-day-old biofilms was 30-fold greater than that of 1-day-old biofilms ($p < 0.001$), which in turn were seven-fold more resistant than planktonic cells ($p < 0.001$). No viable planktonic cells were recovered after exposure to chlorhexidine for 60 min.

Similar differences in the susceptibilities of planktonic cells and biofilms to cetylpyridinium chloride 0.05% solution were recorded. The 5- and 60 min survival levels of cells from 3-day-old biofilms were significantly greater than those for 1-day biofilms and planktonic cells ($p < 0.001$) (Fig. 1).

The same pattern of susceptibility was found for the various cell types exposed to triclosan 0.03% solution for 60 min (Fig. 1); $8.2 \pm 1.6\%$ planktonic cells survived this treatment, which was significantly less than the survival of 1-day-old biofilms ($18 \pm 0.14\%$; $p < 0.001$). These in turn were less resistant than

Fig. 1. Comparison of the effects of: **a**, chlorhexidine digluconate 0.2%; **b**, cetylpyridinium chloride 0.05%; **c**, triclosan 0.03% on 1-day-old planktonic suspensions (▲) and on 1-day-old (■) and 3-day-old (□) biofilms of *A. actinomycetemcomitans*; ○, control diluents.

3-day-old biofilms, which gave an average survival level of $35.0 \pm 28.0\%$. However, because of the wide confidence limits of the latter value, the difference in survival between planktonic and 3-day-old biofilms was not demonstrated to be significant ($p < 0.7$). The differences in survival of the different cell types after exposure to triclosan for 5 min were not significant ($p < 0.5$).

Comparison of antiseptic activities

Consistently, at the concentration used in commercial products, chlorhexidine gave much greater reductions in viable counts of planktonic and biofilm-grown cells than the other two antiseptics (Fig. 1). It was the only antiseptic tested that gave significant ($p < 0.001$) reductions in the viability of planktonic cells and of both types of biofilm after contact for 1 min. Chlorhexidine also gave a $4 \log_{10}$ cycle reduction in the viability of 3-day-old biofilms after exposure for 60 min, compared with decreases of 0.5 and $1 \log_{10}$ units for triclosan and cetylpyridinium chloride solutions, respectively ($p < 0.001$) (Fig. 1). After exposure of 1-day-old biofilms for 5 min, the reductions in viable count compared to controls were $\log_{10} 3.5$ ($p < 0.001$), 1 ($p < 0.001$) and 0.5 ($p < 0.001$) for chlorhexidine, cetylpyridinium chloride and triclosan solutions, respectively (Fig. 1).

Fig. 2 compares the three agents at 0.1% concentration (1.1 mM chlorhexidine gluconate, 2.8 mM cetylpyridinium chloride and 3.5 mM triclosan) against 3-day-old biofilms. It shows clearly that chlorhexidine has the greatest intrinsic antimicrobial action when the antiseptics are compared either as solutions of equal percentage concentration, or when assessed in terms of

Fig. 2. Comparison of the activities of 0.1% concentrations of chlorhexidine digluconate (■), cetylpyridinium chloride (□) and triclosan (▲) against 3-day-old biofilms of *A. actinomycetemcomitans*; ○, control diluents.

molar activity; chlorhexidine 0.1% reduced viability by $3 \log_{10}$ units after 60 min, compared to $1.5 \log_{10}$ units for cetylpyridinium 0.1% ($p < 0.001$). These reductions in viability were significantly greater than the $\log_{10} 0.75$ reduction obtained with triclosan 0.1% ($p < 0.001$).

Concentration of chlorhexidine

The level of activity shown by chlorhexidine, compared to the other antiseptics, underlines the continued interest in its use in the prophylaxis and treatment of plaque-related disease. However, chlorhexidine has an unpleasant taste and, with prolonged use, stains the teeth [12]. Therefore, the effect of reducing the concentration of chlorhexidine on bactericidal activity was investigated. Chlorhexidine 0.1% was almost equally as effective as the commercially used concentration of 0.2% after 1 min ($p < 0.6$) and 60 min ($p < 0.3$) against 3-day-old biofilms (Fig. 3), but gave nearly a 10-fold less reduction in viable count after 5 min ($p < 0.001$). Exposure to 0.05% and 0.01% chlorhexidine gave significantly more survivors than 0.1% at 5 and 60 min ($p < 0.001$). After 1 min, all concentrations of chlorhexidine gave significantly fewer survivors than the control ($p < 0.001$), ranging from $89.7 \pm 6.9\%$ for the 0.01% concentration to $40.0 \pm 7.3\%$ for 0.2% chlorhexidine (Fig. 3).

Activities of pure compounds compared to commercial products

Comparison of the activities of solutions of the three compounds with those of their equivalent commercial product showed each agent to be slightly more effective as a bactericide against 3-day-old biofilms of *A. actinomycetemcomitans* than the commercial product (results not shown). For example, after contact for

Fig. 3. Effect of reducing the concentrations of chlorhexidine digluconate on its activity against 3-day-old biofilms of *A. actinomycetemcomitans*: □, 0.2%, ■, 0.1%, ▲, 0.05%, △, 0.01%, ○, control diluent.

60 min, there were eight-fold more survivors in Corsodyl than in chlorhexidine 0.2% ($p < 0.001$), three-fold more in Merocet than in cetylpyridinium chloride 0.05% ($p < 0.001$), and twice as many in Plax as in triclosan 0.03% ($p < 0.01$). The branded products contain extra ingredients, some designed to improve adherence to the teeth, which increase the residual effect of the mouthwash. However, such formulutory adjuvants may also serve to reduce intrinsic bactericidal activities as determined by the in-vitro test used here.

Discussion

Few data have been published on the susceptibilities of planktonic suspensions of *A. actinomycetemcomitans* to oral antiseptics and no information is available on the susceptibility of biofilms. Reported MICs for chlorhexidine against planktonic cells range between 0.0002 and 0.0125% [13, 14]. In the present study, chlorhexidine was by far the superior agent of the three tested on all growth states of *A. actinomycetemcomitans*, followed by cetylpyridinium chloride, with triclosan being the least active. This order agrees with the activities determined by Moran *et al.* [15] for these three antiseptics against dental plaque.

Results of the present study confirm that bacterial films (especially mature films) are much more resistant to antimicrobial agents than planktonically grown cells. Furthermore, they re-inforce previous recommendations that antiseptic mouthwashes should be tested for activity on inocula grown as biofilms [9, 10, 16, 17]. However, biofilms produced in the laboratory are often less adherent and less virulent than those found in nature [18], which should also be taken into account when trying to achieve in vivo-in vitro correlations. The finding that 3-day-old biofilms of *A. actinomycetemcomitans* were consistently more resistant to oral antiseptics than 1-day-old biofilms may have important clinical relevance. The longer teeth are left uncleaned, the more difficult it will be to inactivate oral bacteria by chemical means [19], and even regular brushing does not remove biofilms completely, particularly from inaccessible crevices.

The concentration of antiseptic and the time of treatment must both be sufficient to ensure adequate antimicrobial action. Commercial mouthwashes are recommended to be retained in the mouth for 1 min and there is some evidence that increasing the volume of mouth rinse improves bactericidal effect [20, 21]. The data presented here suggest that if the retention time could be extended *in vivo* to 5 min, improved activity may result particularly with chlorhexidine and cetylpyridinium chloride (Fig. 1).

The effectiveness of chlorhexidine 0.2% compared to the other two antiseptics suggested that it might be

possible to formulate an effective mouthwash containing a lower concentration, which could be used prophylactically with less risk of tooth discolouration. The data in Fig. 3 are encouraging in this respect: chlorhexidine 0.1% was nearly as efficient as 0.2% against 3-day-old biofilms of *A. actinomycetemcomitans* after exposure for 1 and 60 min, and chlorhexidine 0.1% gives a greater reduction in viability after 5 min than the other two antiseptic solutions tested. However, it would be necessary to conduct similar experiments to compare the activities of chlorhexidine 0.2% and 0.1% against biofilms of other oral pathogens to test this hypothesis further. Wade *et al.* [22] recently reported the anti-staining properties of a novel anti-adherent agent, which could be used in conjunction with chlorhexidine.

Finally, the other additives in commercial mouth rinse products were shown to have a slight, but significant detrimental effect on antimicrobial activity, as judged by the in-vitro test employed. Manufacturers claim that additional substances increase viscosity and so, by prolonging retention of active ingredient on teeth surfaces, may prolong the effective exposure time to at least 5 min after an initial 1-min rinse [23]. This benefit may, therefore, offset any small decrease in intrinsic antimicrobial action [24]. Thus it would appear that testing both pure agents and formulated mouthwashes is necessary to determine whether the interactions of additives reduce potency.

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