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Biofilm development by blastospores and hyphae of *Candida albicans* on abraded denture acrylic resin surfaces

ABSTRACT

Statement of a problem. *Candida albicans* is a known etiological agent of denture stomatitis. Candida hyphae exhibit the ability to respond directionally to environmental stimuli. This characteristic is thought to be important in the penetration of substrata such as resilient denture liners and host epithelium. It has been suggested that hyphal production also enhances adhesion and survival of Candida on host and denture surfaces. Surface roughness can additionally enhance adhesion where stronger interactions occur between cells and surface features of similar dimensions.

Purpose. It is known that cleaning regimens and general use may result in the abrasion of denture acrylic resin. Increased roughness can enhance retention of cells on surfaces. Therefore, the development of hyphal and blastospore biofilms on abraded denture acrylic resin specimens and the ease of removal of these biofilms were investigated.

Material and methods. Biofilms were grown for 48 hours on abraded 1 cm² denture acrylic resin specimens from adhered hyphal phase *C. albicans* or from adhered blastospores. Subsequently, all specimens were stained with Calcofluor White and examined with confocal scanning laser microscopy (CSLM). Biofilms were removed by vortex mixing in sterile phosphate buffered saline (PBS). Removed cells were filtered (0.2 μm pore size). Filters were dried at 37°C for 24 hours for dry weight measurements. Any cells remaining on the acrylic resin specimens were stained with 0.03% acridine orange and examined with epi fluorescence microscopy. Statistical analysis included T-test with a significance value of p=<0.1.

Results. Biofilms grown from both cell types contained all morphological forms of *C. albicans*. Although the underlying surface topography did not affect the amount of biofilm produced, biofilms grown from hyphal phase Candida were visibly thicker and had greater biomass ($P<.05$). These biofilms were less easily removed from the denture acrylic resin, especially in the case of rougher surfaces, evidenced by the higher numbers of retained cells ($P\leq.05$).

Conclusion. The presence of hyphae in early Candida biofilms increased biofilm mass and resistance to removal. Increased surface roughness enhances retention of hyphae and yeast cells and will therefore facilitate plaque regrowth. Minimization of denture abrasion during cleaning is therefore desirable.

INTRODUCTION

Candida albicans is a known etiological agent of chronic erythematous candidosis (denture stomatitis). This inflammatory disorder affects approximately 60% of denture wearers, causing inflammation of the oral mucosa in close contact with the denture.[1] As with natural dentition, dentures provide hard non-shedding surfaces that enable the build-up of plaque biofilms over time. Candida biofilm development on denture acrylic resin begins with adhesion, which can either occur directly to the conditioned surface or via a layer of pre-existing denture plaque.[2] The surface topography of the denture has been shown to greatly influence adhesion and subsequent retention, with more roughened surfaces retaining more organisms.[3-5] The topography of denture surfaces is difficult to regulate. Newly fabricated dentures present a topography reflecting the mucosa of the patient, and are potentially additionally abraded during fabrication and use. Cleaning regimens involving the use of hard brushes or abrasive cleansers may also alter the surface topography and thus may be undesirable.[6]

Candida biofilm formation on denture acrylic resin surfaces has been investigated and described previously.[7] In vitro, attached budding yeast cells (2 to 4 hours) begin to filament after 4 hours, forming pseudo and true hyphae until after 8 hours, neighboring cells and their filaments become entwined, forming spatially organized woven structures. After 24 to 48 hours of undisturbed growth the Candida biofilms increase in complexity, consisting of several different layers and all morphological fungal forms.[8] The ability of *C. albicans* to alter its morphology is considered to be an important contributor to its virulence, [9] with particular focus on hyphal forms. Candida hyphae have been reported to enhance adhesion to surfaces[10] and are known to bind specifically to several human proteins, including fibrinogen, c3d, and lamanin.[11-13] Candida hyphal formation has also been suggested as being important for the invasion of the host epithelium, allowing dissemination of the organism and aiding infection.[14] Recent attention to contact sensing, or thigmotropism, highlights a possible mechanism for this invasion, where hyphal tip extension is directed in response to contact.[15] The effect of substratum surface topography, specifically of denture acrylic resin surfaces, on this phenomenon has not been investigated. Implications for denture hygiene and oral health are also apparent.

Candida morphogenesis and specifically the transition from yeast to hyphal states are considered important factors in virulence. Candida biofilm development on denture acrylic is affected by surface topography, but the effect of topography on different morphological forms of Candida has not previously been investigated. The null hypothesis for the study was that the presence of adhered *Candida albicans* hyphae as opposed to blastospores will not affect the development and removal of subsequent Candida biofilm.

MATERIAL AND METHODS

Culture preparation.

One colony of *Candida albicans* GDH 2346 (NCYC 1467) cultured on Sabouraud's (SAB) Dextrose agar (Oxoid Ltd, Basingstoke, UK) was used to inoculate 100 mL of SAB broth, which was subsequently incubated overnight at 37°C in an orbital shaker (DMS 360, Fisher Scientific, Bishop Meadow, Loughborough, Leicestershire, UK) at 150 rpm. The cells were harvested by centrifugation, washed twice in sterile phosphate buffered saline (PBS) (Oxoid), and resuspended in PBS to an optical density of 1.0 at 540 nm, approximately 1.23 $\pm 0.14 \times 10^7$ cells/mL.

Preparation of surfaces.

Two differently prepared sets of denture acrylic resin specimens were used for the investigation of biofilm growth**.** Two centimeter squared specimens of pink, heat-polymerized polymethyl methacrylate (PMMA. Meadway heat cure polymer/liquid monomer, Bracon dental laboratory products, Etchingham, East Sussex) were produced and abraded by GlaxoSmithKline consumer healthcare (St George's Avenue, Weybridge, UK) using commercially available dentifrices with varying levels of abrasive activity.[16] These surfaces were subjected to the dentifrices with 800 strokes using a soft nylon bristle tooth brush (Oral B, GlaxoSmithKline consumer healthcare, Weybridge, UK) that was rotated 180 degrees after 400 strokes and compressed with a 2.9 N force. Four different degrees of roughened surfaces were used in the investigation: Control (washed with water), low abrasion (washed with Colgate cavity protection 25:40 paste/water ratio. Colgate Ltd, Guildford, UK), medium abrasion (washed with Colgate Total Whitening 25:40 paste/water ratio), and the high abrasion test group (washed with neat Colgate luminous). Following abrasion, these surfaces were rinsed for 30 seconds in running distilled water. These test substrata were used to compare biofilms visually and evaluate biofilm mass.

The second set of surface specimens were made in house (Manchester Metropolitan University) from pink, heat-polymerized PMMA (Bracon dental laboratory products,

Etchingham, UK). These specimens were 1 cm² in size and were abraded manually with p100 (162 µm grit size) emery paper (Wetordry; 3M, Bracknell, UK) with 10 strokes in 1 direction (downwards) parallel to the edge of a ruler.

Hyphal induction and biofilm development.

Forty mL of the standardized cell suspension were added to 4 sterile Petri dishes, each containing 3 replicates of the test materials (PMMA set 1), which were then incubated for 1 hour at 37°C without agitation. After adhesion, test specimens were removed and washed gently by immersion in sterile water and agitation (while immersed) by raising and lowering (parallel to the base of the vessel) 10 times. With sterile forceps, half of the replicates were subsequently placed into sterile 25 mL bottles containing 10 mL SAB broth and incubated for 48 hours at 37°C to produce blastospore biofilms. The broth was replaced with fresh sterile medium after 24 hours. Hyphal production was induced in adhered cells on the remaining half of the test surfaces by incubating them in 50% horse serum ((Oxoid) diluted with sterile water) in a preheated 37°C water bath for 3 hours. Each test specimen was then washed, placed in SAB broth, and incubated at 37°C for 48 hours as above (fresh medium added after 24 hours) to produce hyphal biofilms.

Confocal Scanning Laser Microscopy (CSLM).

After 48 hours incubation, all specimens were removed from the 25 mL bottles and washed as described above, to remove non-adherent cells. Biofilms on surfaces were dried in a class II laminar flow cabinet (BH-EN 2003, Safe lab systems ltd, Bristol, UK) (for 1 hr), stained with 0.5% Calcofluor White diluted with 10% potassium hydroxide (Sigma Aldrich, Steinheim, Germany) and incubated at room temperature in the dark for 45 minutes according to the manufacturer's staining protocol. Biofilms were visualized with the ×40 oil immersion (Type F immersion liquid – Leica, Wetzlar, Germany) lens with Confocal scanning laser microscopy (Leica DM 2005, LCS SPE 1000). Ten fields were examined per test specimen.

Measuring biofilm mass.

In order to assess biofilm mass, individual test surfaces with attached cells were vortex mixed for 30 seconds in 25 mL bottles containing 10 mL of sterile water. The resuspended cells were filtered through 0.2 μm pore filter paper disks (Whatman international ltd, Maidstone, UK), which were then dried for 24 hours in a 37°C incubator and weighed against a sterile filter control. After removal of the biofilms, test surfaces were stained with 0.03% acridine orange (Sigma Aldrich, St Louis Mo) diluted with 2% glacial acetic acid (BDH laboratories, Poole, UK) and examined with epifluorescence microscopy (Nikon Eclipse 6000, Burgerweeshuispad, Amsterdam) for any remaining attached cells. The number of retained cells /hyphae and the percentage of a microscope field covered by cells were determined for each replicate specimen.

XTT assay.

In order to compare the susceptibility of hyphal or blastospore biofilms to denture cleansers, biofilms grown from both cell types on the 1 cm² PMMA surfaces (abraded with p100 grit emery paper) were prepared as previously described and incubated for 1 hour at room temperature in either a denture cleanser (1 tablet [Polident; GlaxoSmithKline consumer healthcare, Weybridge, UK] in 200 mL sterile water at room temperature as directed by manufacturer guidelines) or 200 mL of sterile water at room temperature. The 1 cm² acrylic specimens with attached biofilm were removed and placed into small 5 mL bottles to which 790 μL sterile PBS, 200 μL XTT (Sigma Aldrich) dissolved in PBS to a final concentration of 1mg/mL and filter sterilized with a 0.2 μm pore size filter, and 10 μL Menadione (Sigma Aldrich), prepared in acetone to a 0.44 mM concentration immediately before each assay, was added.[17,18] The 5 mL bottles were incubated at 37°C for 3 hours, allowing the XTT components to interact with the metabolically active cells in the biofilms, releasing a colored formazan by-product into the supernatant. After 3 hours, 200 μL of supernatant from each 5

mL bottle containing each test specimen was transferred to a sterile 96 well microtiter plate (U bottomed) and analyzed for optical density at 492 nm with a microplate reader (Multiskan Ascent; Thermo Lab Systems, Basingstoke, UK).

RESULTS

Biofilms grown from blastospores or hyphae consisted of networks of budding yeast cells, hyphae, and pseudohyphae. However, the biofilms grown from hyphal phase Candida contained longer hyphae compared to the hyphae in biofilms grown from adhered blastospores, which were much shorter and tended to be oriented downwards toward the surface (Fig. 1). Most notably, the biofilms grown from hyphal phase Candida were abundant in branching hyphae that fed through the biofilm structure and often appeared to join at several points, a phenomenon which was not seen as frequently in the blastospore biofilms.

Biofilm Mass

Biofilms grown from adhered hyphal phase Candida had a significantly higher biomass ($P \leq .01$) than those grown from adhered blastospores on control, low, medium, and high abraded test surfaces (Fig. 2). The degree of surface roughness did not significantly affect the biofilm mass. This difference in biofilm mass is visible without magnification (Fig. 3).

Cells remaining on surfaces after washing.

Significantly higher numbers of cells were retained ($P < .01$) on all test substrata for the hyphal biofilms than on the blastospore biofilms (Fig. 4). The difference between hyphal and blastospore biofilm cell retention increased as the level of surface roughness increased.

XTT assay

The hyphal biofilms produced higher spectrophotometer readings ($p \leq .05$) than the blastospore biofilms (Figs. 5, 6) after exposure to either the denture cleanser or water. The

higher readings correspond to higher metabolic activity and thus a higher number of viable cells (or more cell biomass) in the hyphal biofilms than in the blastospore biofims after treatment. After 12 hours incubation, both the blastospore and hyphal biofilms that had been soaked in denture cleanser had reduced activity ($P \leq .05$) where the corresponding biofilms that had been soaked in water remained the same (Figs. 5, 6).

DISCUSSION

The results of this study support rejecting the null hypothesis that the presence of adhered *Candida albicans* hyphae as opposed to blastospores will not affect the development and removal of subsequent Candida biofilm In this study the early presence of Candida hyphae was shown to affect biofilm architecture, increase biofilm mass and reduce the removal of biofilms from denture surfaces. Candida biofilms have been investigated extensively, enabling description of their formation and morphology.[7,18] Specifically, this study aimed to investigate how hyphae influence Candida biofilm development. As has been reported in other studies,[8,18] after 48 hours, biofilms comprise complex and organized matrices containing all fungal cell morphologies. However In this study, adhered Candida hyphae gave rise to biofilms containing more hypha-hypha contact and hyphae were longer and more prevalent throughout the biofilm structure. Candida hyphae have been thought to be more adherent than blastospores;[10] thus an advantage is conveyed in terms of initial colonization as well as in terms of increased biomass. This greater bond and larger amount of mass is likely to confer an advantage, helping to explain why, in this study, the hyphal biofilms were less easily removed from the surfaces than blastospore biofilms. The long branching hyphae running through and across the hyphal biofilms appeared to provide a strong structural framework that increased the resistance to removal of the biofilms and also demonstrated enhanced interactions between hyphae and underlying substratum topography.

The numbers of cells retained on the denture acrylic resin surfaces after the removal of both hyphal and blastspore biofilms increased with increases in surface roughness, with the highest numbers seen on the most abraded surfaces. Similar findings have been reported in previous studies.[3,5] The amount of biofilm on these surfaces following maturation at 48 hours growth however, was not affected by surface roughness. In many healthy denture wearers the accumulation of this biomass is prevented by regular denture cleaning. However even with the most rigorous cleaning regimens, it is unlikely that the denture surface will ever be completely clear of microorganisms. If surfaces that are more roughened retain more Candida following cleaning, these retained cells are able to proliferate upon returning to the oral cavity, the risk of developing mature denture plaque biofilms and subsequent denture stomatits is increased. If the retained cells lose viability, they will nevertheless provide additional attachment sites and nutrient sources for new colonizers. These points are especially of importance to patients who may be more susceptible to infection, or those who are less able to effectively clean their dentures. In addition to this, in this study the difference in retention between the 2 types of Candida biofilm was greatest on the most abraded surfaces, suggesting an increased interaction between hyphae and the larger/more numerous features on that surface. This observation has been made previously[19]; thus, it may be that minimizing denture surface roughness by using non-abrasive or low abrasive cleansing regimens may be a step towards reducing Candida colonization of denture surfaces and denture plaque formation in general.

Biofilms grown from hyphal phase Candida survived better (indicated by the metabolic assay XTT) than those grown from adhered blastospores after exposure to both denture cleanser and water, probably due to the presence of greater numbers of more established hyphae and/or increased biomass. The biofilm phenotype is known to confer various advantages by increasing resistance to antimicrobial challenges and by protecting

cells from mechanical forces.[18,20] The denture plaque biofilm, therefore, poses a challenge to hygiene procedures. There are many protocols available for denture cleaning.[6] Those involving the use of denture cleansers commonly consist of a simple 5 to15 minute soak, followed by a rinse. Brushing is also recommended, to ensure that plaque and debris are removed from the surface. As with these findings, some denture cleansers have been demonstrated to be effective in reducing, but not eradicating Candida biofilms.[21] In this study the cleanser used was tested solely for the effectiveness of its chemical formulation, without effervescence, since the focus of the assay was on viability of the cells in the in vitro biofilm. This enabled comparison of the survival of hyphal and blastospore biofilms. Rinsing was also omitted as these mechanical mechanisms would clearly enhance biofilm removal. Additional study limitations include the omission of a salivary conditioning film, which might affect initial attachment to the surface.. In addition, in vivo, denture plaque comprises a complex mixed microbial population whose structure and physiology might well differ from those of the simple Candida biofilms investigated here. In this study, extended soak times with the denture cleanser of 1 hour and 12 hours were used compared to the 5 minute soak recommended by the manufacturer. Although far removed from the recommended soak time, our parameters might be comparable to an overnight soak: in any case, the work enabled the viability of the different biofilms to be explored further

CONCLUSION

.The presence of hyphae increases the retention of Candida on denture acrylic surfaces and therefore increased its presence in subsequent plaque formed on the surface. More cells were retained on surfaces that were more heavily abraded. Findings have implications in terms of denture hygiene procedures.

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LEGENDS

Fig. 1. CSLM images of *Candida albicans* biofilms (stained with 0.5% Calcofluor White), grown on abraded PMMA. A) biofilm grown from adhered blastospores, B) biofilm grown from adhered hyphal phase Candida. Biofilms grown from hyphal cells are more abundant in long, branching pseudohyphae.

Fig. 2. Mass (g) of biofilms grown from adhered blastospores and hyphal phase *Candida albicans* on PMMA with different levels of surface roughness (control, low, medium, and high abraded) (n=3).

Fig. 3. Biofilms grown on abraded PMMA specimens from attached Candida blastospores (left) and attached Candida hyphae cells (right). Biofilms grown from adherent hyphae (right) are visibly thicker and more textured.

Fig. 4. Number of *Candida albicans* cells retained on differently roughened PMMA specimens (control, low, medium, and high test specimens abraded with dentifrices), after vortex washing for 30 seconds in sterile water (n = 30).

Fig. 5. Effect of soaking in denture cleanser at room temperature for different durations on viability of *Candida albicans* biofilms grown from adherent blastospores or hyphal phase cells (n=3).

Fig. 6. Effect of soaking in water on viability of *Candida albicans* biofilms grown from adherent blastospores and hyphal phase cells (n=3).