Effects of modified pellicles on *Candida* biofilm formation on acrylic surfaces.

Abstract

To examine the role of salivary or serum proteins, such as mucin, fibronectin and mannan-binding protein, the effect of modified pellicles on candidal biofilm formation was investigated in the present study. Supplementation of saliva with FN had no significant effect on biofilm activity. In contrast, the activity of the biofilm formed on either the mucin-coated or FN-coated acrylic was significantly less than that of the control ($p<0.01$). These results suggest that salivary mucin or FN alone does not facilitate the biofilm formation of *Candida*.

In contrast, supplementation of serum with FN increased the biofilm activity of *C. glabrata* as compared with the control. In addition, pretreatment of serum with antiFN monoclonal antibody significantly reduced the biofilm activity of three isolates of *Candida* ($p <0.01$). These results, taken together, suggest that FN together with other serum components such as fibrinogen may facillitate *Candida* biofilm formation. Similarly, pretreatment of serum with antiMBP monoclonal antibody or Con-A seemed to significantly reduce the biofilm activity of some of the *Candida* species ($p<0.05$). Thus it seems that the cell surface mannnan in combination with MBP in serum play an important role in *Candida* biofilm formation.

Our results taken together, suggested that not sole protein predominantly govern the biofilm formation of *Candida,* implying that candidal biofilm formation on acrylic surfaces is a complex phenomenon regulated by a multiplicity of proteins operating intra-orally.

Despite the realization that successful candidal colonization of denture surface is an important step in the pathogenesis of this condition (1), the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood. Indeed components of saliva or serum proteins, such as mucins, fibrinogen and complements specifically bind to *Candida* blastospores and germ tubes (2-5) possibly modifying the process. In recent investigations we have demonstrated the existence of specific interactions between mannoprotein adhesin of *C. albicans* and sugar-moiety of salivary proteins, including mucins, during candidal adherence to protein adsorbed surfaces (6,7), and that salivary and serum pellicles promote fungal colonization on denture lining materials (8). Others have shown that mucinous glycoproteins of human saliva promote adhesion of *C. albicans* to acrylic surfaces by sugar-specific interactions (9). In contrast, some researchers have demonstrated that pretreatment of acrylic strips and/or yeast cells with whole saliva decreases the initial adherence of *C. albicans* to denture acrylic, whereas a serum pellicle promotes adherence (10,11).

Much recently, we suggested that fibronectin, mannan-binding protein, concanavalin-A binding materials should be involved in the fungal biofilm formation on saliva- or serum-coated acrylics (12). However, the role played by these proteins in biofilm formation is not still unclear. In addition, there is no data on the formation of biofilms by non-*albicans* species such as *C. glabrata* and *C. tropicalis*. Thus, the purpose of the present study was to investigate the role of components of saliva or serum proteins in the *Candida* biofilm formation on saliva- and serum-coated acrylic surfaces.

Candida albicans GDH18, *C. glabrata* GDH 1407 and *C. tropicalis* GDH 1362 were used in this study. All isolates were oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and were identified by sugar assimilation test using the API 20C system (API Products, Biomeroux, Lyon, France) and "germ tube" test (13). A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250mM glucose and grown aerobically at 37℃ (6). After overnight culture, the organisms were harvested in the late exponential growth phase, washed twice with 100mM phosphate buffered saline (PBS; pH 6.8) and resuspended to a final concentration of 10^5

cells/ml by using haemocytometeric counts (8,12).

Heat-cured denture acrylic sheets (50 × 50 × 0.7mm) were fabricated according to conventional prosthodontic techniques (11,12). Briefly, denture acrylic poly (methylmethacrylate) powder and monomer liquid (Bio Resin, Shofu, Kyoto, Japan) were mixed according to manufacturer's directions. The mixture was packed into the flask, processed in water tank at 70℃ for 90min and then 100℃ for 30min, according to Japan Industrial Standard (JIS). A smooth surface was obtained by compressing the mixture onto glass slides. The processed acrylic sheets were cut into 10 × 10 × 0.7mm pieces.

Unstimulated whole saliva was collected by expectoration, on ice, from five healthy adult donors (3 males and 2 females) and an equal amount from each donor was pooled. The saliva was clarified by centrifugation at 12,000g, for 15min at 4°C (14). Human serum was purchased from Sigma Chemical Co.(St Louis, MO, USA). Whole saliva and serum were stored at -25℃ before use.

The biofilm assay was conducted as follows. The acrylic strips were coated with protein solutions (described below) by placing them in wells of Multiwell tissue culture plates (Nunclon^{R} Delta, Nunc, Kamstrup, Denmark), into which were dispensed 500 μ l of the protein solution per well, and incubating for 1 hour at 37 °C. After incubation the protein solution was aspirated, 50 μ l of yeast suspension (1×10^{5} cells/ml) was inoculated into each well and the whole assembly incubated at 37°C for 2 hrs to promote yeast adherence and colonization. Subsequently, 2.0 ml of Sabouraud broth was carefully dispensed into each well, and incubated for 72hrs at 37°C. Afterwards each specimen was carefully removed, washed thoroughly by rinsing three times for a total of 60 seconds with distilled water to remove loosely adherent organisms, and the ATP content was measured as previously described (12). The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. All the numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1 % levels.

To examine the effects of several components of saliva on *Candida* biofilm formation, mucin (Bovine submandibular mucin; Sigma Chemical Co., St. Louis, MO, USA; at a conc.

of 2500 μ g/ml), fibronection (FN) (Human serum fibronectin; Sigma Chemical Co., St. Louis, MO, USA; at a conc. of 200 μ g/ml) and saliva supplemented with FN solution (80:20 vol/vol saliva-FN at 200 μ g/ml) was used for the formation of pellicles. As a control pellicle, saliva diluted with PBS (pH 6.8) (80:20 vol/vol mixture) was used.

To examine the role of FN, serum was mixed with (80:20 vol/vol mixture) FN solution (at a conc. of 1000 μ g/ml), and used for the development of the proteinaceous pellicle, as described above. To examine the role of FN or mannan-binding protein (MBP), pretreatment of serum with monoclonal antibody was carried out according to the method of Bouchara et al. (1990;15). Twenty ml of serum was mixed with 2.5ml of PBS (pH 7.4; containing 0.8% NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$) containing 1% bovine serum albumin and either 1:100 diluted mouse antihuman MBP monoclonal antibody (IgG1, 131-1, Serotec) or 1:100 diluted mouse antihuman FN monoclonal antibody (IgG1, 131-1, Serotec) for 30min at room temperature. Each solution was mixed with 2.5 ml of PBS containing 1% bovine serum albumin and 1:10 diluted goat antimouse IgG-gold colloidal particles (E-Y Lab. Inc. San Mateo, CA; particle size 20nm), and incubated for 30min at room temperature. Then each solution was centrifuged with 2000g for 15min, and was stored at -25℃ before use.

Similarly, to examine the contribution of concanavalin-A (Con-A) binding materials to biofilm formation, 20 ml of serum was mixed with 5.0 ml of PBS containing 1% bovine serum albumin and 1:10 diluted Con-A-gold colloidal particles (E-Y Lab. Inc. San Mateo, CA ; particle size 20nm, final protein conc. 3 μ g/ml), for 1hr at room temperature according to the manufacturer's direction. Then each solution was centrifuged with 2000g for 15min, and was stored at -25℃ before use.

To develop a control pellicle for this assay, 20 ml of serum was mixed with 5.0 ml of PBS (pH 7.4; containing 0.8% NaCl, 1mM CaCl2, 1mM $MgCl_2$) for 60min at room temperature, then the serum was centrifuged with 2000g for 15min, and was stored at -25℃ before use (80% diluted serum).

Although several reports have shown the initial suppression of *C. albicans* adherence

on saliva-coated denture surfaces (11,16), others have reported the opposite (9,17). These may reflect the modulation of candidal adhesion due to variables such as the quality of the saliva (18), the yeast isolates and the growth media (10,19,20). Several proteins of serum and saliva have been proposed to specifically bind to *Candida* cells (2-6,15,21,22) and to promote their adhesion (9,11,12,16,17,22,23) as well as biofilm formation (12). Thus the effect of modified pellicles on candidal biofilm formation was examined. *Candida* isolates which demonstrated a high level of biofilm activity in the preliminary study, were chosen for this assay, and biofilm formation by three isolates of *Candida* belonging to three different species, on mucin, FN and saliva supplemented with FN coated acrylic strips was compared with that of diluted saliva coated samples which acted as the control. Supplementation of saliva with FN had no significant effect on biofilm activity ($p>0.05$; Fig. 1). In contrast, the activity of the biofilm formed on either the mucin-coated or FN-coated acrylic was significantly less than that of the control ($p<0.01$; Fig. 1). These results suggest that salivary mucin or FN alone does not facilitate the biofilm formation of *Candida*, though both proteins have been reported to mediate the adhesion of *C. albicans* through specific adhesin-receptor interactions (9,22,24). One reason for the suppression of the biofilm activity of *Candida* by these proteins may be their occupation and saturation of the receptor sites on the *Candida* cell surface with resultant down regulation of intercellular co-adhesion leading to biofilm formation. On the other hand, as shown in our previous study, *Candida* cell colonized in multiple layer with hyphal emergence on saliva-coated acrylics (12), however sparse colonization in monolayer was observed on FN-coated acrylics in the present study (data not shown). Thus lack of morphlogical changes and cell-cell interactions via salivary protein, such as the blastospore-blastospore aggregation should be attributed to the reduced activity of biofilm formed on FN-coated acrylics.

In contrast, supplementation of serum with FN increased the biofilm activity of *C. glabrata* as compared with the control. In addition, pretreatment of serum with antiFN monoclonal antibody significantly reduced the biofilm activity of three isolates of *Candida* (*C. albicans* GDH 18, *C. glabrata* GDH 1407 and *C. tropicalis* GDH 1362) ($p<0.01$). These results, taken together, suggest that FN together with other serum components such as

fibrinogen may facilitate *Candida* biofilm formation, although FN by itself does not appear to promote the process. Similarly, pretreatment of serum with antiMBP monoclonal antibody or Con-A seemed to significantly reduce the biofilm activity of some of the *Candida* species ($p < 0.05$; Fig. 2). Thus it seems that the cell surface mannnan in combination with MBP in serum play an important role in *Candida* biofilm formation.

To conclude, our results tend to suggest that not sole protein predominantly govern the biofilm formation of *Candida,* implicating that the mechanisms of *Candida* biofilm formation should be complex, including a multiplicity of interactions between cell-proteins and protein-protein in the components of proteinaceous pellicles of biological fluids such as saliva or serum.

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Legends to Figures

Fig. 1

Biofilm activity of 3 isolates of Candida spp. on mucin (mucin; 2500 μ g/ml), fibronection(FN; 200 μ g/ml) and saliva supplemented with FN solution (sal+FN) coated acrylic specimens. As a control pellicle, saliva diluted with PBS (pH 6.8) (sal) was used. The assays were carried out on two independent occasions, with quadruplicated samples on each occasion.

Fig. 2

The effects of pretreatment of serum on biofilm formation of Candida. (See materials and methods for details.)

Se + PBS: Control pellicle, serum (80%) was mixed with PBS (20%).

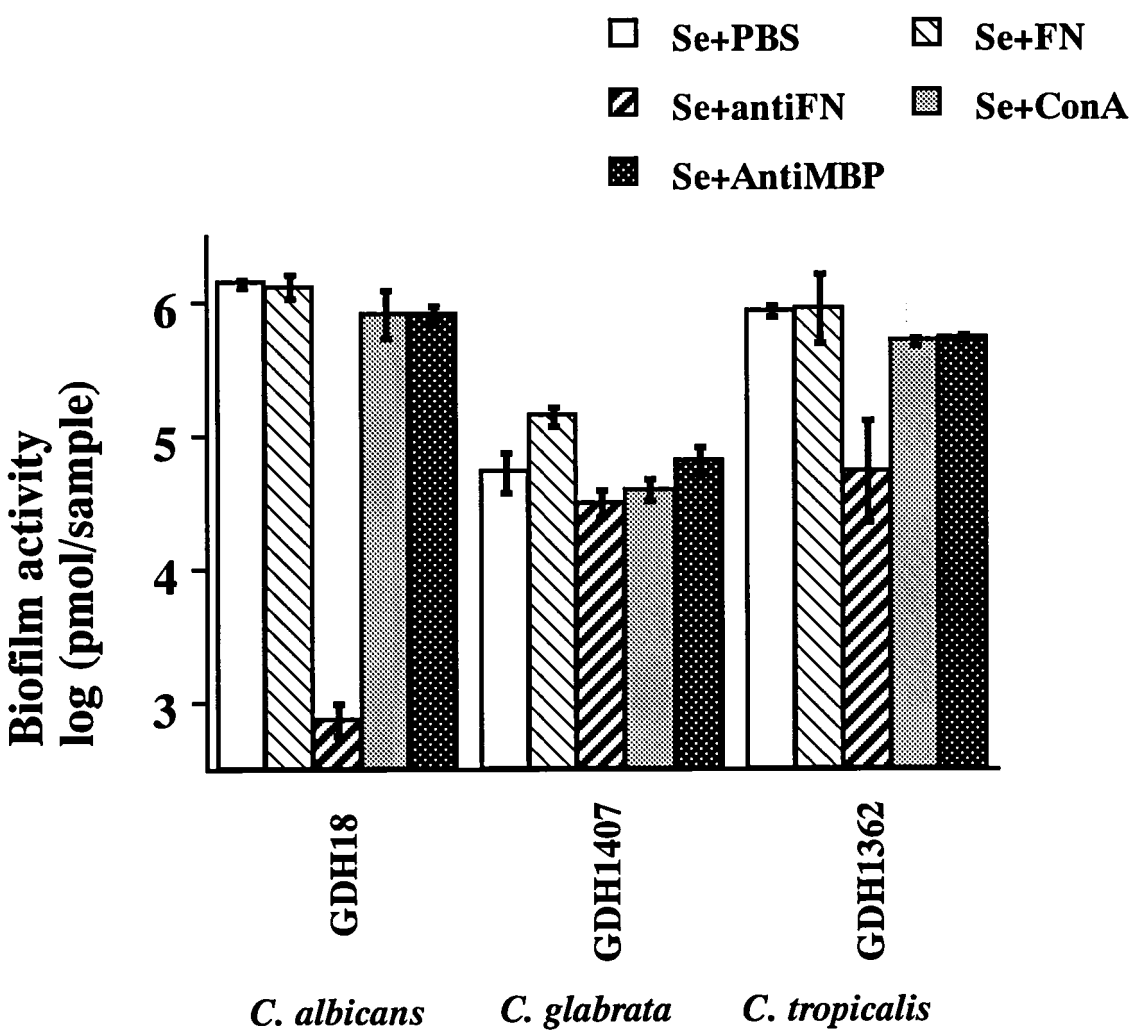
Se + FN: Serum (80%) was mixed with FN (20%) solution (1000 μ g/ml).

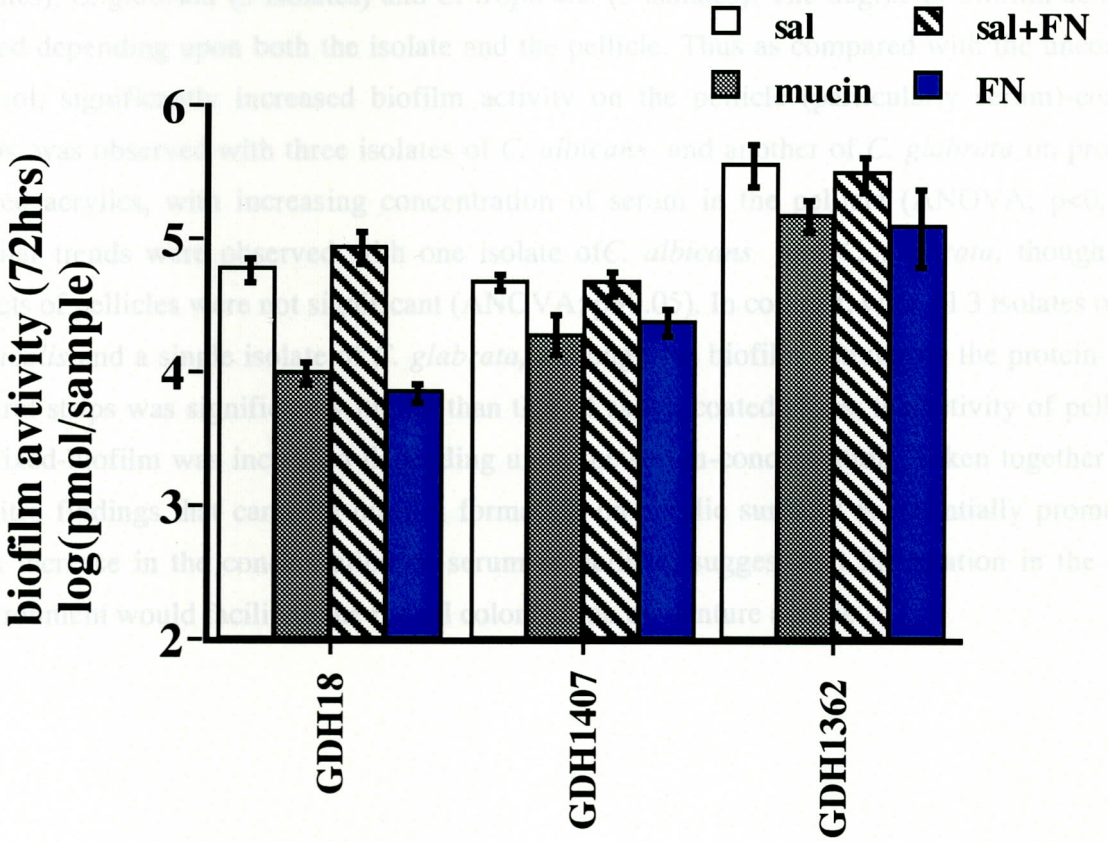
Se + antiFN: Serum pretreated with anti FN moloclonal antibody.

Se + anti MBP: Serum pretreated with anti MBP monoclonal antibody.

Se + ConA: Serum pretreated with ConA.

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion.


Se+PBS
Se+FN
Se+antiFN
Se+ConA
Se+AntiMBP
Biofilm activity
log (pmol/sample)
6
5
4
3
GDH18
GDH1407
GDH1362
C. albicans
C. glabrata
C. tropicalis


sal
sal+FN
mucin
FN
biofilm avtivity (72hrs)
log(pmol/sample)
6
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GDH18
GDH1407
GDH1362
C. albicans
C. glabrata
C. tropicalis