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The Role of Saliva and Serum in *Candida albicans* Biofilm Formation on Denture Acrylic Surfaces

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The long term effect of either a salivary or a serum pellicle on *Candida albicans* biofilm formation on denture acrylic surfaces was investigated both by quantifying the ATP (adenosine triphosphate) content of the resultant biofilms and by scanning electron microscopy. When the biofilm formation on saliva-coated acrylic strips was examined, the yeasts initially colonised this surface at a slower rate than the controls although with increasing incubation time, at 72 h, the ATP content was almost ten-fold higher than the protein-free control strips. Ultrastructural studies revealed this to be due to cell aggregation and hyphal emergence, phenomena not observed in the controls. As compared with the control strips, biofilm activity of the serum-coated strips was almost 100-fold greater within 48 h incubation, and scanning electron microscopy revealed multilayer blastospore-blastospore co-adhesion, germ tube, hyphal and pseudohyphal emergence and blastospore-hyphal coadherence. Further immunocytochemical observation revealed that concanavalin-A binding material and fibronectin were involved in biofilm formation on both saliva and serum coated specimens and, in addition, mannan-binding protein and protein-A binding material also contributed to the biofilm formation on serum coated specimens.

KEY WORDS: *Candida albicans*; biofilm; saliva; serum; fibronectin; mannan-binding protein.

INTRODUCTION

Candida albicans is frequently recovered from denture fitting surfaces which act as a reservoir of infection in *Candida*-associated denture stomatitis (syn. chronic atrophic candidiasis).[4,9] Although successful candidal colonisation of the denture surface has been recognised as an important step in the pathogenesis of this condition,[32] the role played by saliva or serum pellicles during the colonisation process is poorly understood.

Components of saliva or serum proteins, such as mucins, fibrinogen and complement, specifically bind to *Candida* blastospores and germ tubes.[2,6,7,29] Specific interactions between mannoprotein adhesin of *C. albicans* and sugar-moiety of salivary proteins, including mucins, have also been demonstrated during candidal adherence to protein adsorbed surfaces,[22,23] and it has been shown in our studies that salivary and serum pellicles may promote fungal colonisation on denture lining materials.[24] Others have shown that mucinous glycoproteins of human saliva promote adhesion of *C. albicans* to polymethylmethacrylate by sugar-specific interactions.[10]

In contrast to these observations, a number of workers have demonstrated that pretreatment of acrylic strips and/or yeast cells with whole saliva decreased the initial adherence of *C. albicans* to denture acrylic, whereas a serum pellicle promoted adherence.[18,19,34]

It is therefore evident that the relationship between the salivary or serum pellicle on denture surfaces and candidal colonisation has been a subject of controversy. This may be attributed in part to the diverse and complex attributes of saliva and/or serum, which modulate clearance, aggregation and adherence of microorganisms, and also to various microbial factors, such as specific and non-specific interactions, cell-growth and coadhesion,

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which are involved in fungal colonisation of surfaces. Further, there are little data on the long term activity of *Candida* biofilms on mucinous surfaces once the initial attachment process is complete and it is not known whether the quality of the primary mucinous layer (i.e. saliva or serum) affects the subsequent growth and colonisation of the adherent yeasts.

For the current experiments we have adapted a bioluminescent adenosine triphosphate (ATP) assay based on firefly luciferase-luciferin system, which is known as a simple and convenient method for accurate enumeration of viable cells[15] and has been used previously for gram-positive and gram-negative bacterial biofilm.[1,11] The purpose of the present study was to analyse in detail the role of the salivary and serum pellicles in the initial and subsequent stages of *C. albicans* biofilm formation on acrylic surfaces. Preliminary immunocytochemical studies were also performed to investigate the nature of the *Candida* biofilm on protein-coated surfaces.

MATERIALS AND METHODS

Microorganisms and growth conditions

Candida albicans GDH 18 and GDH 19, oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and School, were used in this study. The isolates were identified by sugar assimilation test using the API 20C system (API Products, Biomeroux, Lyon, France) and 'germ tube' test.[36] A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250 mM glucose and grown aerobically at 37°C.[22] After the overnight culture, the yeast was harvested in the late exponential growth phase, washed twice with sterilised distilled water and resuspended to a final concentration of 10^5 cells/ml by a spectrophotometeric and haemocytometric means.[24]

Fabrication of acrylic strips

Heat-cured denture acrylic sheets (50 × 50 × 0·7 mm) were fabricated according to conventional prosthodontic techniques. 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°C for 90 min and then 100°C for 30 min, 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 × 0·7 mm pieces.

Saliva and serum

Unstimulated whole saliva was collected from a healthy donor and clarified, according to the method of Gibbons *et al.*[12] with modification, by centrifugation at 12 000 *g* for 15 min at 4°C. Human serum was purchased from Sigma Chemical Co. (St Louis, MO, USA). Whole saliva and serum were stored at −25°C before use.

Cell growth and ATP (adenosine triphosphate) analysis

To examine the interrelation between yeast growth and adenosine triphosphate (ATP) content, 100 µl of yeast suspension, 250 µl of either saliva or serum and 4·0 ml of Sabouraud dextrose medium were dispensed into a series of sterile glass tubes. In the control tubes, saliva or serum was replaced by an equal volume of sterile distilled water. These tubes were incubated at 37°C for 6, 12, 24, 48, 60, 72, 96, 120, 144 and 168 h. After each of these intervals, the tubes were carefully vortexed, 1·0 ml of each sample was mixed with 4·0 ml of the reagent containing benzalkonium which extracts intracellular ATP[37] and allowed to react for 5 min in an ultrasonicator. Then the solution was clarified by filtration (pore size 0·45 µm) and the amount of ATP was quantified using a bioluminescence apparatus (ATPA-1000, TOA Electronics Ltd, Tokyo, Japan).[1] This apparatus used the firefly-luciferase system to determine the concentration of cellular ATP and is based upon the measurement of light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. In this system, the light intensity is directly proportional to the concentration of ATP.[37] A conventional growth curve was constructed by haemocytometer counting of yeasts in the control cultures, which did not exhibit either germ tube or hyphal formation. The relationship between the yeasts/ml and ATP content was subsequently established.

Assay procedures

The colonisation assay was conducted as follows. The acrylic strips were coated with saliva or serum by placing them in wells of Multiwell tissue

culture plates (NunclonR Delta, Nunc, Kamstrup, Denmark), into which were dispensed 500 µl of the protein solution per well, and incubating for 1 h at 37°C. Saliva or serum was substituted with an equal volume of sterile distilled water in the control wells. After incubation the protein solution was aspirated, 50 µl of yeast suspension (1×10^5 cells/ml) was inoculated into each well and the whole assembly was incubated at 37°C for 2 h to promote yeast adherence and colonisation. Subsequently, 2·0 ml of Sabouraud broth was carefully dispensed into each well, and incubated for 0, 6, 12, 24, 48, 60, 72, 96, 120, 144 and 168 h at 37°C. Afterwards each specimen was carefully removed, washed thoroughly by rinsing three times for a total of 60 s with distilled water to remove loosely adherent organisms, immersed in 5·0 ml of the extraction-reagent (benzalkonium; 37) and allowed to react for 5 min with ultrasonication. The resultant reagent solution was then filtered (pore size 0·45 µm) to clarify, and subjected to ATP-measurement. To monitor the yeast growth, the total amount of ATP in each well was also quantified.

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. All the numerical data obtained were analysed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1 per cent levels.

Ultrastructural observations

For ultrastructural studies, specimens were removed from the wells after the assay, washed with sterile distilled water and fixed in 2·5 per cent glutaraldehyde and 1·0 per cent osmium tetroxide. Afterwards they were dehydrated through a graded series of ethyl alcohol (50–90 per cent), immersed in t-butyl alcohol (three times for 10 min) stored at 4°C. Each specimen was then freeze dried, sputter coated with a layer of gold to a thickness of 15–20 nm and observed under a Scanning Electron Microscope (JMS-6300, Joel, Tokyo, Japan) using standard procedures.

Immunocytochemical observations

For concanavalin-A staining, each specimen was removed from the wells after the biofilm assay (168 h), washed with 10 mM PBS (pH 7·4; containing 0·8 per cent NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and immersed in PBS containing 1 per cent bovine serum albumin and 1:10 diluted concanavalin-A-gold colloidal particle (E-Y Lab. Inc., San Mateo, CA; particle size 20 nm, final protein concentration 3 µg/ml) for 1 h at room temperature according to the manufacturer's direction.

To examine whether IgG or IgM is involved in fungal biofilm formation or not, each specimen was removed from the wells after the assay (168 h), washed with 10 mM PBS and immersed in PBS containing 1 per cent bovine serum albumin and 1:10 diluted protein-A-gold colloidal particle (E-Y Lab. Inc., San Mateo, CA; particle size 30 nm) for 1 h at room temperature according to the manufacturer's direction.

To observe the localisation of fibronectin or mannan-binding protein, immunocytochemical staining was carried out according to the method of Bouchara *et al.*[3] Each specimen was removed from the wells after the assay, washed with 10 mM PBS and immersed in PBS containing 1 per cent bovine serum albumin and either 1:100 diluted mouse antihuman mannan-binding protein monoclonal antibody (IgG1, 131-1, Serotec) or 1:100 diluted mouse antihuman fibronectin monoclonal antibody for 30 min at room temperature. Each specimen was then washed with PBS and incubated in PBS containing 1 per cent bovine serum albumin and 1:10 diluted goat antimouse IgG-gold colloidal particle (E-Y Lab. Inc., San Mateo, CA; particle size 20 nm) for 30 min at room temperature.

Specimens were next washed with PBS, fixed in 2·5 per cent glutaraldehyde and dehydrated as described above. Each specimen was then sputter-coated with a layer of gold to a thickness of 10 nm[38] and observed under a Scanning Electron Microscope (JMS-6300, Joel, Tokyo, Japan) using standard procedures.

RESULTS

The preliminary experiments were performed to establish the relationship between *C. albicans* growth in saliva or serum supplemented Sabouraud medium, or the latter alone (control), and the ATP content. As enumeration of yeasts in the saliva and serum supplemented media was difficult due to mixed blastospore and hyphal phase growth, the number of yeasts in the control tube (which grew solely in the blastospore phase) was used to correlate the cell growth and the ATP content of the test media.

As shown in Figure 1 a to c, there was a good correlation between the yeast numbers or the yeast

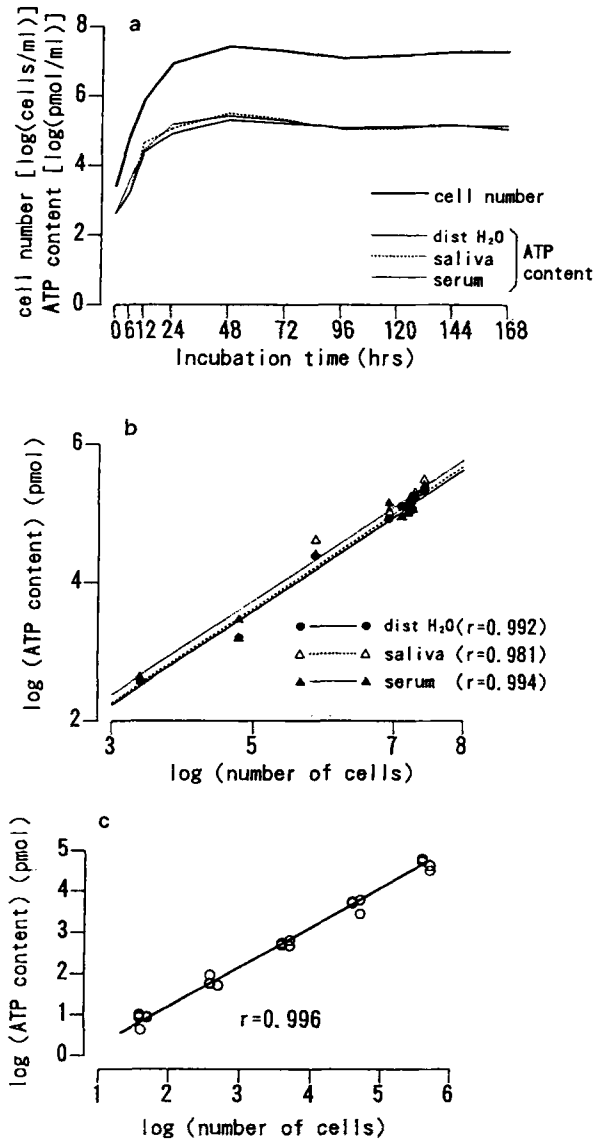

Figure 1. (a) The growth curve of *C. albicans* GDH 18 in the control (Sabouraud medium), saliva- and serum-supplemented media as assessed by ATP measurement and haemocytometric cell counting. (b) The relationship between the cellular ATP content and haemocytometric cell counts of *C. albicans* GDH 18 in the control (Sabouraud medium), saliva- and serum-supplemented media. Each experiment was repeated on two independent occasions with quadruplicated determinations on each occasion. (c) The relationship between the cellular ATP content and haemocytometric cell counts of *C. albicans* GDH 18 at the ranges of 40–10^5 cells

growth in all three media and the ATP content. Typical growth characteristics of *C. albicans* were observed in the saliva/serum supplemented media and the controls, with increase in cell numbers up to 48 h and reaching a plateau phase thereafter.

No significant difference in the ATP content was observed either in the test or the control suspensions, at any incubation interval (GDH 18; ANOVA; $P>0{\cdot}05$). On regression analysis a highly significant positive correlation was observed between the ATP content of the yeasts and the cell numbers, in the test samples with saliva or serum or, the control samples (GDH 18; Figure 1 b,c; Student t-test; $P<0{\cdot}01$, $r=0{\cdot}98$–$0{\cdot}99$). Similarly, a significant correlation between the ATP content of the yeasts and the cell numbers, in the test samples with saliva or serum, or in the control samples, was also observed with *C. albicans* GDH 19 (data not shown). In all the experiments, the extracellular ATP content in each sample was less than 0·5 per cent of intracellular ATP, indicating the reliability of the ATP measurement.

As these experiments indicated that the ATP content was directly proportional to the number of yeasts, irrespective of their morphology or the growth phase, the former parameter was utilised to evaluate the long term growth characteristics and biofilm formation of *C. albicans* on saliva- and serum-coated acrylic strips.

The effect of different protein pellicles coating the denture material on the growth of these isolates in the suspending medium, and the biofilm development on acrylic strips are shown in Figure 2 a,b. As expected, the yeast growth curves of the test and the control specimens (containing acrylic strips) described an almost identical path comparable to the foregoing preliminary study (Figure 2 a,b). Thus, in all three suspensions (containing the control, saliva- and serum-coated strips), a rapid phase of growth was observed during the first 12 h, which steadily levelled off to a plateau after 48 h, beyond which no further growth was perceptible.

When the *C. albicans* GDH 18 biofilm activity on the control and serum-coated strips was compared by the ATP assay method, the activity increased rapidly up to 12 h at an exponential rate, closely resembling the yeast growth curves in the suspending medium. However, the increase in the biofilm activity of the saliva-coated strips was relatively slow (GDH 18; Figure 2 a). The increase in the biofilm ATP content on the saliva- and serum-coated strips continued steadily up to about 72 h although the control biofilm activity peaked after 12 h, reaching a plateau thereafter (Figure 2 a), with a slight decrease in recorded values.

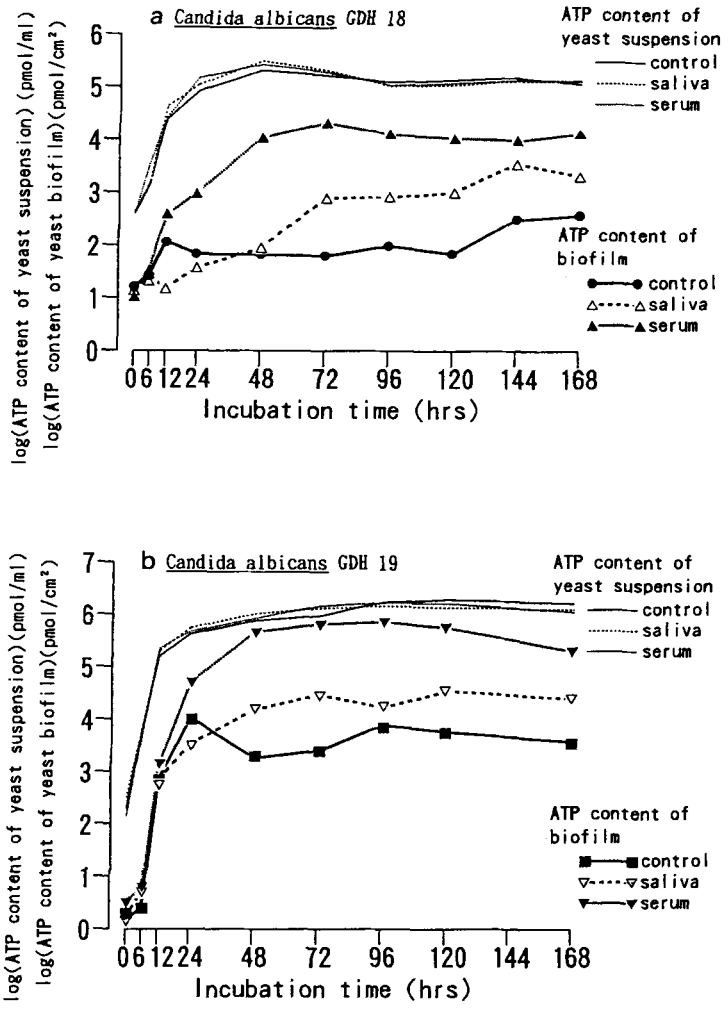

Figure 2. (a) The growth curve and biofilm activity of *C. albicans* GDH 18 on the control (uncoated, saliva- and serum-coated acrylic strips in Sabouraud medium as assessed by ATP analysis. The test samples of acrylic were coated with a pellicle of either saliva or serum prior to incubation. (b) The growth curve and biofilm activity of *C. albicans* GDH 19 on the control (uncoated), saliva- and serum-coated acrylic strips in Sabouraud medium as assessed by ATP analysis. The test samples of acrylic were coated with a pellicle of either saliva or serum prior to incubation. Each experiment was repeated on two independent occasions with quadruplicated determinations on each occasion

As to the *C. albicans* GDH 19, the biofilm activity on the control, saliva- or serum-coated strips increased rapidly up to 12 h at an exponential rate, resembling the yeast growth curves in the suspending medium. Although the rate of increase in the biofilm activity in the saliva-coated strips slowed down earlier than its counterparts (GDH 19; Figure 2 b), the biofilm ATP content on both the saliva- and serum-coated strips increased steadily up to about 72 h. In contrast, the peak activity of the control biofilm was observed after 24 h (Figure 3 a), decreased significantly at 48 h, and plateaued thereafter.

When the relative ATP content of the 24 h and 72 h biofilms of both *C. albicans* GDH 18 and GDH 19 were compared, a serum pellicle was significantly more effective in promoting fungal biofilm formation than a saliva pellicle (24 h and 72 h, ANOVA; $P<0{\cdot}01$). Interestingly, in the case of *C. albicans* GDH 18, saliva pellicle suppresses

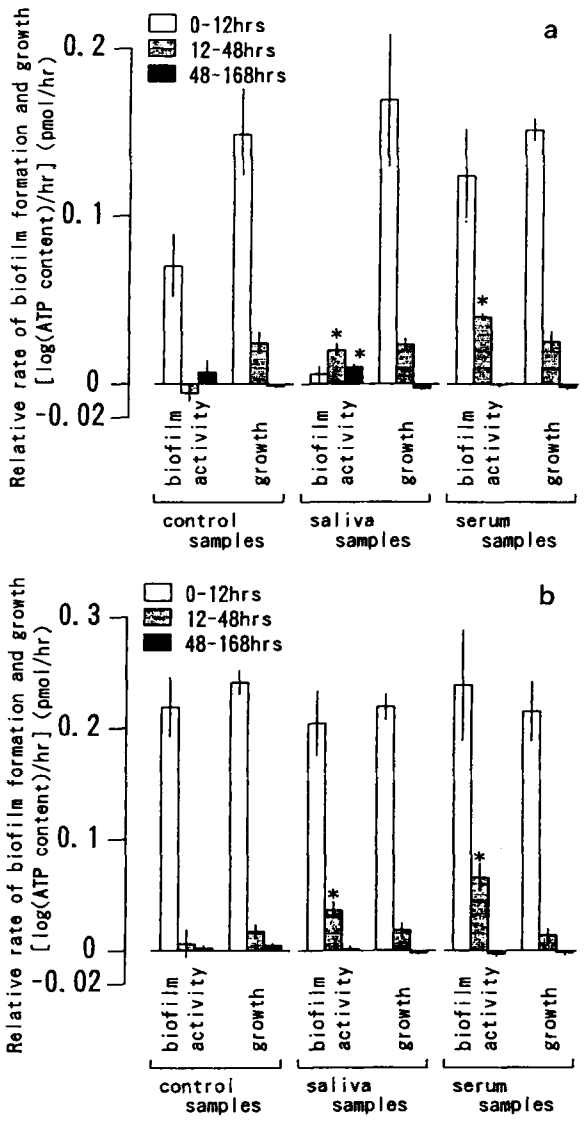

Figure 3. (a) Comparison of *C. albicans* GDH 18 biofilm activity on the control (uncoated), saliva- and serum-coated acrylic strips and the growth rates on the respective specimens during the initial (0–12 h), mid (12–48 h) and late (48–168 h) phases of the incubation periods. Asterisks refer to the significant differences between the control and test samples ($P<0.05$). (b) Comparison of *C. albicans* GDH 19 biofilm activity on the control (uncoated), saliva- and serum-coated acrylic strips and the growth rates on the respective specimens during the initial (0–12 h), mid (12–48 h) and late (48–168 h) phases of the incubation periods. Asterisks refer to the significant differences between the control and test samples ($P<0.05$)

or shows no effect on biofilm formation when compared with the protein-free control specimen, during the initial stages of colonisation (i.e. 12–24 h, ANOVA; $P<0.01$). None the less this behaviour pattern changes as the biofilm matures. Thus, at 72 h the biofilm on the serum-coated strips exhibited more than 100-fold increased cellular kinetics than the control, uncoated strips, and the activity on the saliva-coated strips exceeded that of the controls by a factor of ten.

The relative growth rate of yeast suspension and biofilm formation rate of *C. albicans* GDH 18 during the initial (0–12 h), mid (12–48 h) and the final (48–168 h) phases of the study were further compared (Figure 3 a). In the absence of a protein pellicle (control samples) the activity of the biofilm slows down considerably after 12 h as opposed to yeasts on the saliva/serum-coated specimens, although during the final phase of the study the reverse was observed. In the pellicle coated samples the biofilm metabolism was relatively higher or similar during the mid phase of the experiment (12–48 h, Figure 2) when compared with yeast growth rate in either the test or the control suspensions. The saliva biofilm demonstrated a significantly high activity during the final phase (48–168 h) of the study when compard with either the serum-coated ($P<0.01$) or control samples ($P<0.05$), or the growth parameters in any of the suspensions ($P<0.01$) (Figure 3 a).

Results similar to *C. albicans* GDH 18 were obtained when the growth rate of yeast suspensions and biofilm formation characteristics of *C. albicans* GDH 19 were compared, except that, during the initial period of the study (0–12 h), no significant differences in the foregoing parameters were observed between the control, saliva- or serum-coated specimens (Figure 3 b).

SEM Observations

The processes of biofilm formation was similar with both *C. albicans* isolates. During the initial phase, small numbers of *C. albicans* blastospores were seen sparsely attached to both the control and saliva-coated strips and hyphal emergence was seldom observed (Figure 4 a,b). in contrast, a large number of blastospores with and without germ tubes colonised the serum-coated specimens, though the proportion with germ tubes was very low during this period (Figure 4 c). During the mid to late phase (12–168 h), the control specimens were sparsely colonised with *C. albicans* blastospores (Figure 4 d), these exhibited blastospore-blastospore coadhesion on longer incubation (not shown). During the latter period of the study a large population of colonised yeasts also

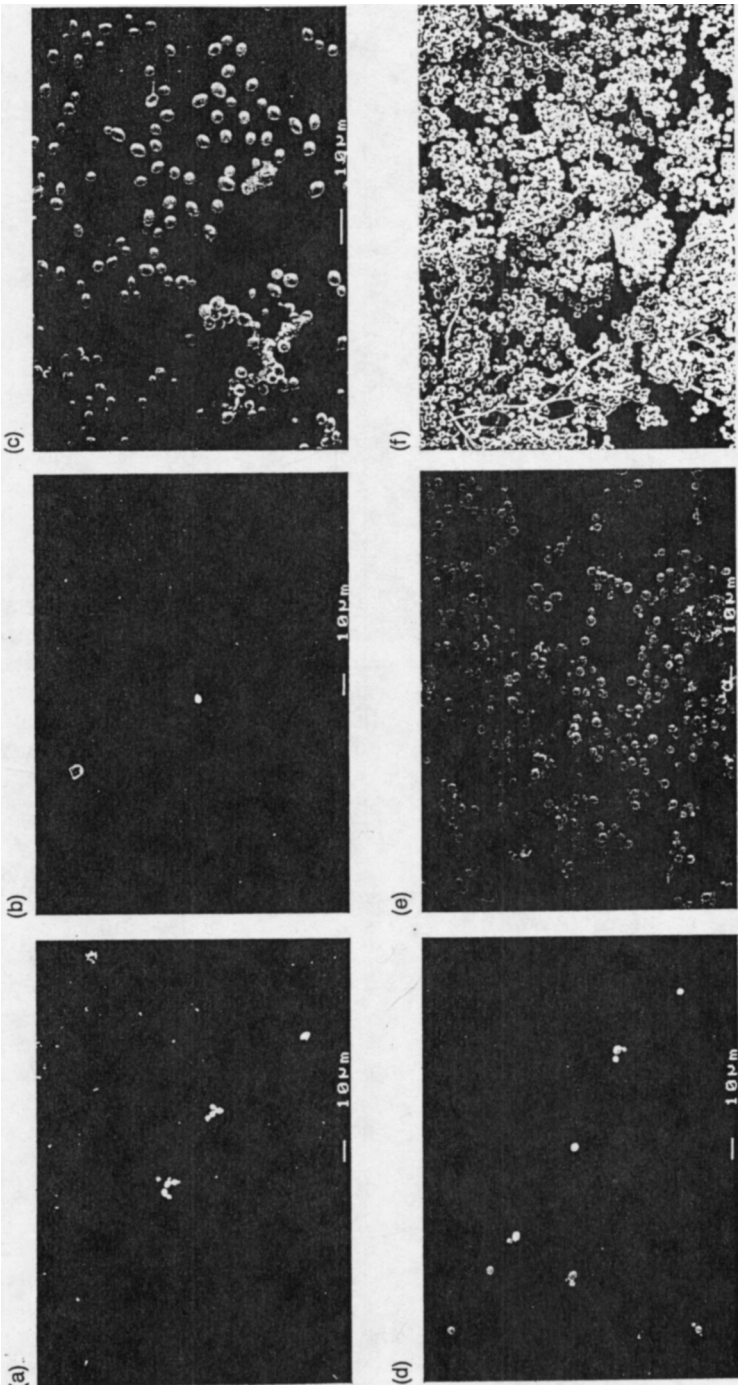

Figure 4. Scanning electron micrographs of the initial phase (12 h) of *C. albicans* GDH 18 colonisation on the control (a), saliva- (b) and serum-coated (c) acrylic strips and the late phase (72 h) of colonisation on the control (d), saliva- (e) and serum-coated (f) acrylic strips

demonstrated blastospore-blastospore co-adhesion on the saliva-coated specimens (Figure 4 e), and, in addition, hyphal emergence and profuse biofilm formation was also observed in the serum-coated specimens (Figure 4 f). The latter population comprised a dense network of yeasts, germ tubes, relatively young hyphae, and pseudohyphae (Figures 4 f and 5 a to c). Further observation revealed that the extracellular matrix mediated the blastospore-blastospore and/or blastospore-hyphae co-adhesion on both the saliva- and the serum-coated acrylic strips (Figure 5 a). In addition, hyphae in contact with the surface of acrylic strips occasionally produced strandlike adhesive material, which has not been previously reported (Figure 5 b,c). The nature of this surface materials which contribute to biofilm formation was evaluated using Con-A, Protein-A, anti-fibronectin monoclonal antibody and anti-mannan binding protein monoclonal antibody.

With *C. albicans* GDH 18, Con-A-gold colloidal staining revealed that saliva- or serum-biofilms were more reactive to Con-A binding as compared with control biofilms (Figure 6 a to c). Further, ultrastructural studies indicated that fibronectin is involved in the biofilm formation on saliva- or serum-coated specimens (Figure 6 d,e) and that human mannan-binding protein and protein-A binding material (perhaps IgG and IgM) may contribute to fungal biofilm development on serum-coated specimens (Figure 6 f,g). Control specimen of both isolates were unreactive with any of these materials (Figure 6 a), except for control specimen of *C. albicans* GDH 19 which reacted with Con-A.

DISCUSSION

A number of experimental approaches have been made to examine the mechanisms of *C. albicans* adherence to solid surfaces, such as denture acrylic.[8,10,17,20,30,32,39] Even the earliest investigators of this topic, using visual quantification of adherent yeasts, reported the high affinity of *C. albicans* to denture acrylic and modulation of this attachment process due to saliva and serum pellicles.[31,33] Although the latter groups observed the initial suppression of *C. albicans* adherence on saliva-coated denture surfaces,[32] others have reported the opposite effect.[39] While these may reflect the modulation of candidal adhesion due to variables such as the quality of the saliva,[38] the yeast isolates and the growth media, it should also be noted that the phenomenon of adherence may represent only the first step in the colonisation process[14] which, as time progresses, leads to a formation of a thin biofilm and then a multilayer, climax community of plaque.

A deeper understanding of adherence and the subsequent behaviour of *Candida* biofilms requires first, the examination of sequential samples over a period of time and, more importantly, a method for accurate quantification of yeast biofilm formation which may exhibit dimorphic growth patterns as well as co-adhesion, aggregation and multilayer growth over a prolonged period of colonisation.[24] The main impediment for such research to date has been the lack of a relatively simple assay system to accurately quantify the cell growth. Recently, Hawser and Douglas[13] reported fungal biofilm formation on catheter material by using dry weight, colorimetric (MTT) and radiometric assays. However, in preliminary studies, we found that the dry weight and MTT assay methods were less sensitive in detecting the early stage of yeast colonisation. Further the results of MTT assay were affected by the salivary and serum proteins (data not shown). Hence we adopted another assay system, bioluminescent ATP assay, which has previously been used to measure bacterial biofilm activity.[1,11] Farber and Wolff[11] have conclusively shown that the result obtained by this ATP assay was consistent with that obtained by either conventional viable counts or radiolabelling methods. Using this method we noted an excellent correlation between the yeast cells and the ATP content in either the test or the control samples. This was not surprising as the assay was based on the fundamental principles of ATP analysis which states that the amount of cellular ATP correlates with the dry weight, the volume and the number of viable cells,[35] irrespective of the morphological attributes of the yeast.

It is known that some salivas suppress candidal growth while others do not[33] and hence it is possible that our observations on the lack of effect of saliva on fungal growth (Figures 1 and 2) could either be due to the quality of the saliva used and/or its high dilution in the incubation medium. A similar explanation could be offered for growth in serum containing medium as the latter is known to affect candidal cell kinetics in a variety of ways.[25] These findings confirm the results of our previous study where pellicles of salivary or serum proteins coating denture material did not essentially affect the rate of fungal growth in the

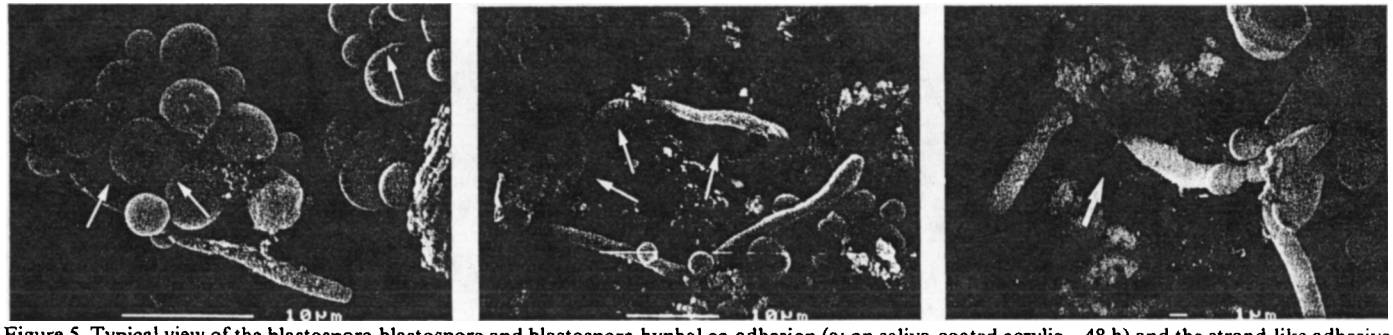

Figure 5. Typical view of the blastospore-blastospore and blastospore-hyphal co-adhesion (a: on saliva-coated acrylic—48 h) and the strand-like adhesive material mediating the hyphal adhesion to acrylic surface (b,c: on serum-coated acrylic—168 h). Arrows indicate the presence of extracellular, and strand-like polymeric adhesive material

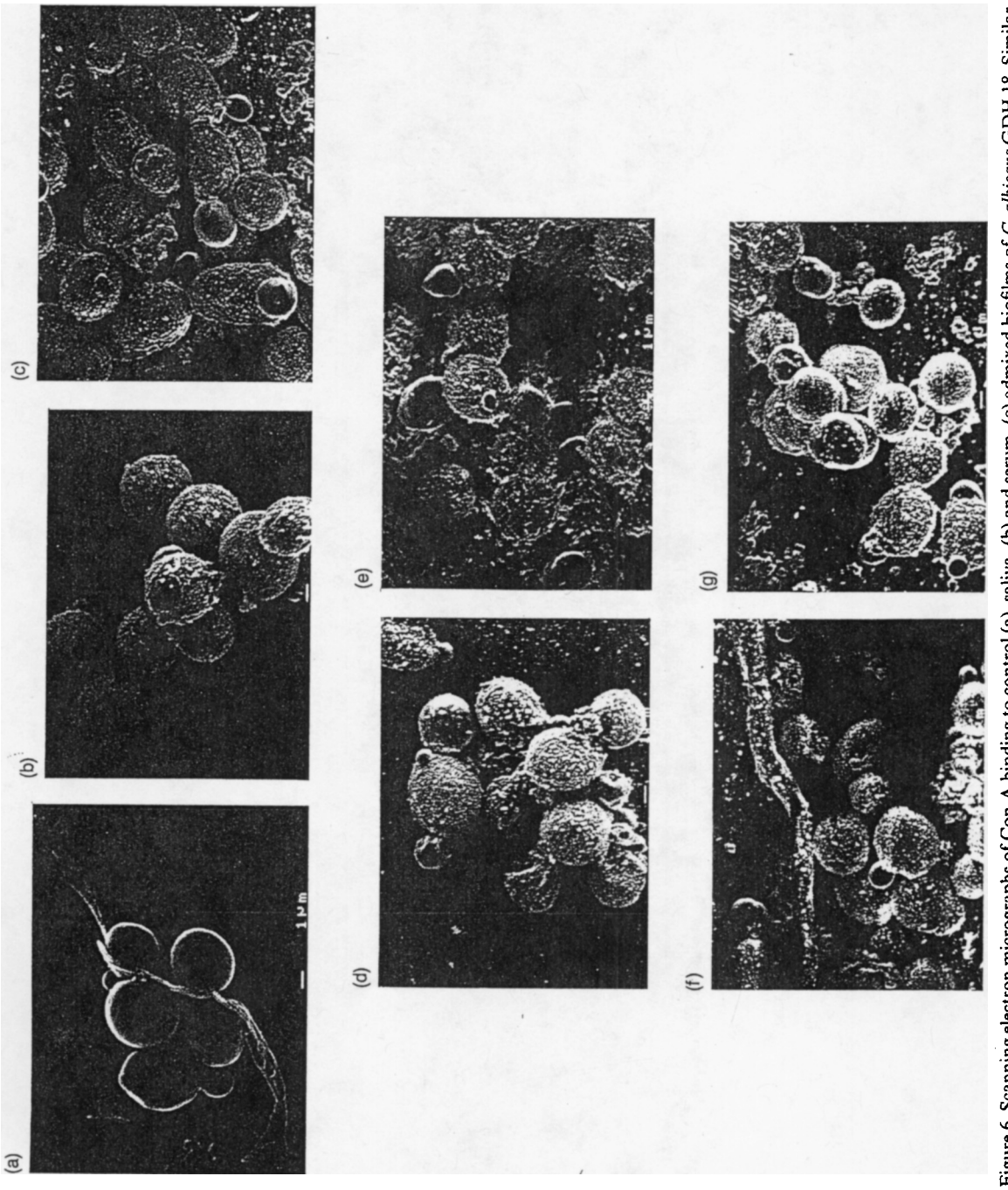

Figure 6. Scanning electron micrographs of Con-A binding to control (a), saliva- (b) and serum- (c) admixed biofilms of *C. albicans* GDH 18. Similar views of anti-fibronectin antibody binding to saliva- (d) and serum- (e) admixed biofilms of fungi. Anti mannan-binding protein antibody (f) and protein-A (g) binding to serum-admixed biofilm of fungi. All controls were similar to control biofilm shown in Figure 6 a

suspending medium, as monitored by pH alterations.[24] Further studies however are required to substantiate the current observation and to clarify the interactions between these oral fluids and candidal growth on solid substrates.

As opposed to these growth correlates, the activity of the yeast biofilms on the pellicle coated acrylic strips varied considerably. Thus, during the primary colonisation phase (0–12 h) the activity of the saliva-biofilm of *C. albicans* GDH 18 increased slowly as compared with the control and the serum-coated strips (ANOVA; $P<0{\cdot}01$); the latter exhibiting the greatest activity. However this phenomenon was not as clear cut in *C. albicans* GDH 19, irrespective of protein coat. These results are consistent with the observations of Samaranayake *et al.*[32] who reported that a mixed salivary pellicle, compared to a serum pellicle, retards yeast adhesion to acrylic and those of Vasilas *et al.*[39] and Edgerton *et al.*[10] who reported promotion of candidal adhesion due to salivary pellicles.

Experiments with a firmly adsorbed pellicle simulate only an incomplete and static picture of the oral environment as fungal colonisation and subsequent biofilm formation is a complex phenomenon. Interactions occur not only between the pellicle and the organisms but also with the constituents of the solid-liquid interface involving saliva or serum which may modify phenomena such as coaggregation and coadhesion. As the described ATP quantification method allows such studies, the biofilm assay was carried out under conditions in which both adsorbed and free, saliva or serum proteins existed. From these long term studies it would seem that a mixed salivary pellicle may play a protective role in suppressing candidal biofilm formation on acrylic surfaces at least during the intitial (up to 12 h) colonisation stages.

In contrast, the biofilm on serum-coated specimens developed to a greater extent during the 12 h incubation period (Figures 2 and 3) suggesting that a pellicle comprising serum proteins may facilitate initial colonisation and subsequent development of a candidal biofilm. These findings are in accordance with previous studies.[24,32] Further, SEM observation revealed this phenomenon to be partly related to the emergence of germ tubes (Figure 4)—appendages which are thought to produce adhesins for plastic surfaces[37] and possess binding sites for several serum protein components such as fibrinogen, fibronectin and complement factors.[2,6,7,27] In addition, the observation that blastospore-substrate adhesion occurred to the greatest extent on the serum-coated specimens (Figure 4 c) implies that another type of adhesin-receptor-like mechanisms may be involved in the presence of serum components. Further studies are required to elucidate this phenomenon.

During the 12–48 h incubation period which corresponds to the late exponential to early stationary growth phase, the biofilm formative activity progressed steadily in both the saliva- and serum-coated specimens, though the activity of the control strips reached a plateau after a 12 h or a 24 h period (Figures 2 a and 4). This implies a limited activity of the control biofilm, devoid of either saliva or serum, a fact which was confirmed by ultrastructural studies where sparse yeast colonisation comprising exclusively of blastospores was noted (Figure 4 a). Furthermore, in serum-coated specimens which demonstrated the highest biofilm activity throughout the 72 h period, multiple yeast layers of blastospores, germ tubes, pseudohyphae and hyphae were seen with blastospore-blastospore, blastospore-hyphal co-adhesion (Figure 4 f) and hyphal adhesin expression (Figure 5 b,c). This was in contrast to saliva-coated strips which exhibited predominantly the blastospore-blastospore co-adhesion, and occasional blastospore-hyphal co-adhesion (Figures 4 e and 5 a). Particularly noteworthy was the extracellular material which has been reported to mediate the adherence of both blastospore and hyphae to either vaginal or skin epithelial cells.[28,29] Although this extracellular material mediating blastospore-blastospore and blastospore-hyphal co-adhesion appears to be similar to mannoprotein-like materials reported by Hawser and Douglas,[13] it was not observed in control biofilms of *C. albicans* GDH 18, in the absence of proteins (Figure 6 a). However, Con-A binding material (perhaps mannan or mannoprotein) was observed in control biofilms of *C. albicans* GDH 19 (not shown).

We believe that this is the first account of the 'strand-like' adhesins mediating hyphal adhesion. Though not chemically defined by the present assay, the different appearance of hyphal adhesin might reflect the existence of proteins coating the substrates, since Ray and Payne[29] have suggested that such material may account for the glycocalyx of *Candida*. Further characterisation of this adhesive material is required.

Our results also show that both Con-A binding material and fibronectin are involved in *Candida* biofilm development with saliva and serum (Figure 6 b to e). Further, the material observed in

serum-biofilms also reacted with antimannan-binding protein and protein-A (Figure 6 f,g), suggesting that biofilm formation is a complex phenomenon involving glycoproteins, fibronectin, mannan-binding protein and perhaps IgG or IgM. In addition, yeasts in control biofilms were not reactive to Con-A, whereas yeasts in saliva- or serum-samples were more reactive to the reagent. Since the production of a large amount of mannan layer are known to depend on the growth conditions, such as high concentrations of galactose, it is possible to offer two explanations; firstly, an interaction with the sugar moieties of proteins which adsorbed on to cell surfaces and, secondly, the possibility that the yeasts may have digested salivary or serum proteins, or their sugar moieties, with the resultant appearance of a mannan layer. Either or both of these mechanisms may be involved in the observed phenomena.

This is the first report suggesting that several components of serum, particularly fibronectin, are involved in yeast biofilm development. Interestingly, fibronectin is known to interact specifically with other serum components, such as fibrinogen or fibrin, which has been reported to bind specifically to *Candida* cell surfaces.[2,,27] Further, Negre *et al.*[21] have elegantly demonstrated the mechanism of the binding of soluble fibronectin to *C. albicans* and the adherence of this yeast to fibronectin-coated acrylic surfaces. While the foregoing amplifies our findings, the specific mechanisms which entail the interactions need to be clarified. This is clinically relevant as fungal biofilm formation on catheter materials exposed to body fluids, particularly blood or serum, causes serious disease.

In clinical terms, our results imply that an acquired salivary pellicle on denture surface will modify the yeast colonisation process and initially, at least, will retard biofilm formation. These results tend to support the work of Olsen and Haanes[26] who showed increased yeast colonisation of the upper acrylic plates in monkeys with reduced salivary flow. Furthermore, *Candida*-associated denture stomatitis is a common complaint among patients with Sjogren's syndrome whose salivary flow is absent or minimal.[16] Similarly, it is believed that trauma to the palatal mucosa is an important co-factor in the pathogenesis of *Candida*-associated denture stomatitis,[5] and the current findings illustrate how a resultant serum exudate may potentiate the disease process by significant enhancement of the activity of the candidal biofilm, over an extended period.

To conclude the ATP assay method we have described for investigating candidal biofilm formation is a simple, reproducible means by which the activity of this common fungal pathogen on solid substrates—such as implants, contact lenses and catheter surfaces, in addition to acrylic—could be explored under varying environmental conditions. While our results tend to suggest that mixed saliva and serum may modify the biofilm formation via multiple factors such as co-adhesion, aggregation, germ tube or hyphal emergence and adhesin expression, the method could be used as a valuable tool in investigating parameters such as the long term effect of antifungal agents on candidal biofilm formation.

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