MYCOLOGY

Candida albicans isolates from HIV-infected and AIDS patients exhibit enhanced adherence to epithelial cells

S. P. SWEET, SHARON COOKSON and S. J. CHALLACOMBE

Centre for the Study of Oral Manifestations of HIV Infection, Department of Oral Medicine and Pathology, UMDS Guy's Hospital, London SE1 9RT

Summary. The increased prevalence of oral candidosis associated with HIV infection must be intrinsically related to immunological changes in the host, but might also involve alterations to the infecting strains of yeast. This study aimed to determine if strains of *Candida albicans* isolated from asymptomatic HIV-infected individuals or AIDS patients possessed altered adherence properties in an in-vitro buccal epithelial cell (BEC) adherence assay. *C. albicans* isolates from 49 patients with HIV infection or AIDS adhered to BEC in significantly higher numbers than isolates from 49 control subjects ($p < 0.001$). No significant differences in adherence were detected between strains isolated from HIV-infected or AIDS subjects, or between strains isolated from *C. albicans* carriers (low salivary *C. albicans* counts) or subjects with oral candidosis. The presence of whole saliva significantly inhibited the binding of candida to BEC ($p < 0.001$), but the significant difference in adherence between the HIV/AIDS and control isolates was maintained. The effect of saliva was independent of salivary candida antibodies and was abolished by treatment with protease or neuraminidase, suggesting the involvement of salivary mucins. The results of this study suggest that HIV infection is associated with the selection of strains of *C. albicans* with an increased ability to adhere to oral mucosa.

Introduction

Candida species are carried as part of the commensal oral flora of c. 40% of healthy individuals, normally without disease.¹ However, various factors can predispose to candidosis, including diabetes, pregnancy, antibiotic therapy and, in particular, HIV infection. Candidosis is the most common oral manifestation of HIV infection and is recognised as an important indicator of HIV disease and its progression. The first documented patient with AIDS displayed symptoms of oral candidosis² and it has been demonstrated that the frequency of isolation of *Candida* spp. and clinical signs of oral candidosis increase with advancing HIV infection,^{3,4} making it an important predictive sign for the subsequent development of AIDS.⁵

Adherence to an oral surface is essential to initiate the process of colonisation and infection.⁶ Therefore, the binding of *C. albicans* to the oral mucosa must be a pre-requisite for infection and may be regarded as the first step in the process culminating in oral candidosis. Numerous studies have addressed the question of the

mechanisms involved in the complex process of yeast adhesion to epithelial cells and other surfaces. A number of investigators have concentrated on identifying the terminal sugars of the host cell surface glycoproteins that interact with the fungal cell wall.⁷ Sugars such as L-fucose, N-acetylglucosamine, mannosamine, glucosamine and galactosamine were found to inhibit adhesion.^{7,8} Also, a number of putative adhesins of *C. albicans* have been studied including cell-wall chitin,^{9,10} lipids,¹¹ complement receptors,^{12,13} mannan,^{14,15} proteins^{16,17} and in particular mannoproteins.^{18,19} Evidence suggests that mannoproteins of *C. albicans* are the principle adhesins responsible for adhesion to host cells, and these have been partially characterised.^{18,20,21} Other biophysical factors have also been seen to greatly influence yeast binding to epithelial cells, including cell-surface hydrophobicity,^{22,23} pH, carbon dioxide and iron concentrations.^{24–26} These studies allude to the adherence of *C. albicans* as a specific, multifactorial process involving several types of candidal cell-surface adhesins and various binding sites on host cells.

Recently it has been suggested from DNA fingerprinting,^{27–29} karyotyping³⁰ and phenotyping³¹ studies that HIV infection might be associated with the

selection of *C. albicans* strains resulting in replacement of the original commensal strains. It is feasible that this process is related to the selection of strains with altered virulence determinants leading to colonisation with *Candida* populations that are more able to cause disease in immunologically compromised hosts. If this process of selection is indeed related to pathogenicity, it may be possible to measure alterations in virulence determinants, such as the ability to adhere to oral mucosal tissues. Therefore, the aims of this study were to determine if strains of *Candida* isolated from patients with HIV infection and AIDS possess altered adherence properties in an in-vitro buccal epithelial cell (BEC) adherence assay.

Materials and methods

Source of *C. albicans* isolates

Strains of *C. albicans* were isolated from 49 HIV antibody-positive patients and 49 control subjects. The HIV-infected subjects consisted of 41 patients with no AIDS-defining illnesses with a mean age of 35 years (range 20–58), and eight AIDS patients with a mean age of 42 years (range 24–61). The control subjects consisted of 49 patients with a mean age of 43 years (range 20–78) attending an oral medicine clinic and presenting with various oral pathologies such as xerostomia, recurrent oral ulceration or lichen planus, and not known to be at risk of HIV infection. The control patients were selected as a group with an increased susceptibility to oral candidosis but without evidence of immunosuppression. The majority of the asymptomatic HIV-infected patients and control subjects and two of the AIDS patients had no history of antibiotic or antimycotic therapy in the previous year, and none was receiving antibiotics or antimycotics at the time of sampling. Saliva cultures indicated that 22 of the 41 HIV patients, five of the eight AIDS patients, and 30 of the 49 control subjects had high yeast counts indicative of oral candidosis (> 1000 cfu/ml of saliva).

Isolation of *C. albicans*

Yeasts were isolated from 100 μ l of whole saliva on Sabouraud's dextrose agar (SAB) as described previously.³² Yeast cfu were counted after incubation for 2 days at 37°C. Representative colonies were subcultured and *C. albicans* isolates were selected after identification with the API 20 AUX system (biomerieux, Marcy l'Etoile, France), chlamydospore production on corn-meal agar and germ-tube production in serum. Yeasts were stored as SAB stab cultures at 4°C and in glycerol 40% at –20°C.

Candida suspensions

Suspensions were prepared from *C. albicans* grown on SAB agar for 48 h at 37°C. A single colony was

suspended in 10 ml of phosphate-buffered saline (PBS), pH 7.2 and washed three times by centrifugation at 2500 *g* for 10 min. The yeast cell concentration was determined with a haemocytometer and adjusted to 10^7 cells/ml.

Buccal epithelial cells (BEC)

BEC were collected from healthy laboratory personnel with no signs or symptoms of oral candidosis or other oral pathology. None was taking antibiotics at any time during the study. Some experiments were performed with BEC from six males with a mean age of 31 years (range 23–37) and six females with a mean age of 27 years (range 22–30). However, the majority of experiments were done with BEC from a 22-year-old female.

BEC were collected, at the same time each day, by gently rubbing the buccal mucosa with a sterile cotton wool swab. Cells were dislodged from the swab by agitating in 10 ml of PBS, pH 7.2. The cells were passed through 70- μ m pore-size filters (Becton Dickinson, Lincoln Park, NJ, USA) to remove sheets and clumps of cells, then washed three times in PBS by centrifugation at 2500 *g* for 10 min before resuspending to a concentration of 10^5 cells/ml, measured with a haemocytometer.

Adherence assay

The adherence assay used has been described previously.²⁶ Briefly, 100- μ l samples of BEC and yeast cell suspensions (100 yeasts:1 BEC) were mixed in sterile plastic 7-ml screw-capped bottles and incubated with shaking at 37°C. After 45 min, 5 ml of PBS were added to each bottle to minimise further attachment. BEC with attached yeasts were collected on 25-mm diameter, 12- μ m pore-size polycarbonate filters (Nuclepore, High Wycombe) and washed twice with 5 ml of PBS to remove unattached yeast cells. The filters were then air dried and Gram stained. The number of yeasts adhering to 100 BEC was determined by light microscopy at a magnification of $\times 400$. *C. albicans* NCPF 3153 was included in each batch of assays as an internal control. Assays were done in duplicate and data were analysed by an unpaired *t* test.

Addition of saliva

BEC and *C. albicans* were suspended in a 1 in 10 dilution of whole saliva obtained from the BEC donor. Saliva was clarified by centrifuging at 20000 *g* for 10 min. In addition, some saliva samples were depleted of *C. albicans* antibodies by adsorbing saliva with formalin-fixed *C. albicans* NCPF 3153 cells according to the method of Coogan *et al.*³² Adsorbed saliva contained no measurable levels of *Candida*-specific antibodies as determined by ELISA.³² As a control, saliva samples were also adsorbed with formalin-fixed *Escherichia coli*. Some saliva samples were also incubated at 37°C with protease (pronase E; Sigma) to a

final concentration of 0.05 mg/ml for 1 h, then proteolysis was stopped by the addition of a one-tenth volume of 0.05 mM phenylmethylsulphonyl fluoride (PMSF). Saliva was also incubated at 37°C for 1 h with neuraminidase (Sigma) 20 units/ml, followed by heating at 56°C for 1 h to inactivate the enzyme.

Results

Adherence of C. albicans from HIV-infected subjects, AIDS patients and controls

The mean numbers of adherent *C. albicans* were significantly higher with the isolates from HIV-infected and AIDS patients compared with the control isolates ($p < 0.001$; fig. 1). The HIV and AIDS isolates adhered in similar numbers and demonstrated a mean 40% increase in adherence compared with the control isolates. The addition of whole saliva significantly inhibited the binding of the HIV, AIDS and control *C. albicans* isolates ($p < 0.001$) by a mean of 33, 44 and 62%, respectively. The significant difference in adherence between the HIV or AIDS isolates and the control isolates was still evident regardless of the presence of saliva in the assay system ($p < 0.001$; fig. 1). A previous history of antibiotic or antimycotic use within the previous year by the HIV and AIDS patients had no effect on adherence.

Candidal carriage versus candidosis

Analysis of the adherence data according to whether isolates were obtained from patients with oral candidosis ($> 10^3$ cfu/ml saliva) or from patients with

Fig. 1. Adherence of *C. albicans* strains isolated from controls, HIV-infected or AIDS patients to BEC suspended in PBS with no saliva (▨); or in PBS with a 1 in 10 dilution of saliva (□). Bars represent the SEM. * Significant difference in mean adherence levels of the HIV and AIDS isolates compared with the control strains ($p < 0.001$). † Significant difference in mean adherence levels of the HIV, AIDS and control isolates suspended in PBS compared with a 1 in 10 dilution of saliva ($p < 0.001$).

Fig. 2. Adherence of *C. albicans* strains isolated from controls, HIV-infected or AIDS patients with low levels of candidal carriage (▨) (carriers; < 1000 cfu/ml of saliva) or with candidosis (□) (> 1000 yeast cfu/ml of saliva), to BEC suspended in PBS with saliva (1 in 10 dilution). Bars represent the SEM. * Significant difference in mean adherence levels of the HIV and AIDS isolates compared with the control strains ($p < 0.001$).

Fig. 3. Adherence of *C. albicans* strains isolated from control or HIV-infected patients to BEC from male (□) or female (▨) donors, and suspended in PBS (no saliva). Bars represent the SEM. * Significant difference in mean adherence levels of the HIV isolates compared with the control strains ($p < 0.001$).

low salivary *C. albicans* counts ($< 10^3$ cfu/ml) showed no significant differences in mean levels of adherence between the two groups (fig. 2). This was evident with the HIV, AIDS and control isolates, and in the presence or absence of saliva.

BEC from male and female donors

Adherence assays were performed with six HIV and six control *C. albicans* isolates and with BEC obtained from six female and six male subjects. There were no significant differences in mean adherence levels between the male and female BEC, with either the HIV or control isolates (fig. 3). The increased adherence of

Fig. 4. Adherence of a control isolate of *C. albicans* to BEC suspended in PBS or in PBS with 1 in 10 dilutions of saliva, adsorbed saliva depleted of *C. albicans* antibodies, protease-treated saliva or neuraminidase-treated saliva. Bars represent the SEM. * Significant difference in mean adherence levels with saliva or adsorbed saliva compared with no saliva or protease- or neuraminidase-treated saliva ($p < 0.001$).

the HIV isolates, noted with the previous experiments with a single female donor, were maintained with BEC from all of the donors.

Inhibition of adherence by saliva

The mechanism of salivary inhibition of adherence was investigated with a single control strain of *C. albicans*. Addition of saliva to the assay system produced a significant decrease in adherence of 64% ($p < 0.001$; fig. 4). Substitution of antibody-depleted saliva previously adsorbed with formalin-fixed *C. albicans* (or *E. coli*), showed similar levels of inhibition of adherence (fig. 4). In contrast, treatment of saliva with either protease or neuraminidase eliminated the inhibitory effects of saliva (fig. 4).

Discussion

The proposed requirement for adhesion as the first step in the process of colonisation and infection of a host has led to the development of a number of adherence assay systems. A widely used model to study the adherence of *Candida* spp. and other oral micro-organisms utilises an in-vitro buccal cell adhesion assay. However, this method has limitations. For example, the receptiveness of both buccal and vaginal epithelial cells for *C. albicans* can vary significantly between donors and from day-to-day with the same donor.³³⁻³⁵ It has been postulated that the hormonal status of the donor may influence adherence,³⁴ and that fluctuations in hormone levels in menstruating females may affect the adherence of *C. albicans* to vaginal epithelial cells.³⁶ More recently Theaker *et al.*³⁷ reported that adherence of *C. albicans* to buccal cells also varied with changes in the menstrual cycle, although this conclusion was based on BEC from only one female donor. A retrospective analysis of adherence data from this study showed no

influence on candidal adherence due to the hormonal cycles of the female donor used for most of the present study. Furthermore, no significant differences in adherence were found with BEC obtained from male or female donors (fig. 3).

The results of this study suggest that HIV infection and AIDS is associated with the selection of strains of *C. albicans* with an enhanced ability to adhere to BEC (fig. 1). The HIV and AIDS *C. albicans* isolates adhered in similar numbers, and nearly all of these strains adhered better than all of the control isolates. The presence in the HIV group of healthy, asymptomatic patients suggests that the selection of *C. albicans* strains with altered adherence properties occurs early in the course of HIV infection, before symptoms of immune deficiency are evident and prior to the sequelae of opportunist mucosal infections and consequent treatment regimens. Therefore, the apparent selection of *C. albicans* in HIV infection appears to be independent of antifungal or antibiotic use or other treatments. The selection of more adherent *C. albicans* may contribute to the predisposition of HIV-infected individuals to oral candidosis; however, increased adherence did not correlate with the presence of oral candidosis (fig. 2), suggesting that, as might be expected, other factors must also act to predispose the host to infection. The influence of HIV infection on other virulence determinants of *C. albicans* remains to be determined, as does the role of HIV-induced immunosuppression *versus* immunosuppression due to other causes.

The enhancement of adhesion in HIV infection and AIDS was also evident in the presence of saliva, despite up to 60% inhibition of adherence, suggesting that this process may be functional *in vivo*. Saliva was approximately twice as effective at inhibiting adherence of the control *C. albicans* compared with the HIV and AIDS isolates, indicating that the apparent selection of *C. albicans* in HIV infection may relate to avoidance of the adherence-moderating effect of saliva. Whilst micro-organisms must adhere to an oral surface to avoid the flushing action of saliva, host responses are aimed at limiting adherence and colonisation. Saliva contains various components with antifungal activity, including lysozyme, lactoferrin, lactoperoxidase, calprotectin and histidine-rich polypeptides that may help to control oral candidal populations.³⁸ Alterations to salivary composition or to mucosal tissues as a result of HIV infection may provide the selective pressures necessary to induce alterations in the populations of colonising micro-organisms.

Salivary immunoglobulin A (IgA) has been implicated in the inhibition of candidal adherence to host surfaces and may function primarily by binding to the surface of specific micro-organisms and interfering with cell-to-cell interactions.³⁹ The role of immunoglobulins or other specific factors in the inhibition of adherence was investigated with saliva samples adsorbed with suspensions of formalin-fixed *C.*

albicans (fig. 4). Adsorbed saliva samples contained no measurable IgA by ELISA. However, both adsorbed and unadsorbed saliva showed a significant inhibition of *C. albicans* binding, suggesting that the components in saliva able to inhibit *C. albicans* binding in this assay are probably not antibody related. These results contradict previous studies showing that a decrease in anti-candida titres due to immunoprecipitation of IgA from saliva correlated with increased adherence.³⁹ However, the protective function of salivary IgA in HIV infection has been questioned. HIV-infected patients with oral candidosis have been shown to produce increased levels of salivary candida IgA antibodies, indicating that secretory antibodies are produced in response to infection, but episodes of oral candidosis frequently recur.³²

Non-specific salivary components may also

influence adhesion interactions. Salivary glycoproteins have been shown to inhibit the attachment of oral streptococci to buccal cells,⁴⁰ but mucins may also promote the adherence of streptococci to solid surfaces⁴¹ via neuraminidase-sensitive sialic acid residues.⁴² Comparatively few studies have investigated interactions between *Candida* spp. and salivary components, although the salivary constituents bound by *C. albicans* have been shown to consist predominantly of low mol.-wt mucins.⁴³ The observation that treatment with neuraminidase or protease effectively abolished the ability of saliva to inhibit candidal adherence (fig. 4) concurs with the implication of mucinous glycoproteins as adherence modulating factors.

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