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Differential invasion of *Candida albicans* isolates in an *in vitro* model of oral candidosis

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The study assessed the ability of *Candida albicans* isolates to invade an *in vitro* oral tissue model. The extent and pattern of isolate invasion was then correlated with the infection origin of the isolate to identify characteristics that may be restricted to specific forms of oral infection, particularly chronic hyperplastic candidosis (CHC). Reconstituted human oral epithelium was infected with *C. albicans* isolated from normal oral mucosa ($n = 4$), CHC ($n = 7$), non-CHC oral candidoses ($n = 4$) and squamous cell carcinoma (SCC; $n = 4$). After infection for 24 h, histological analysis revealed yeast adhesion, hyphal extension, and invasion of the epithelium. Differential patterns of invasion were evident and, whilst consistent for a given isolate, did not relate to the infection origin of the isolate. Two principal patterns of invasion were evident and described as either a 'localised' or a 'uniform' distribution of invading hyphae. Several isolates also exhibited superficial infection with limited hyphal invasion. In conclusion, the use of the *in vitro* tissue model allowed the assessment of the invasive capabilities of isolates of *C. albicans*. However, the apparent differences in invasive characteristics did not appear to be related to the clinical origin of isolates.

Key words: *Candida albicans*; oral candidosis; tissue invasion

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The opportunistic pathogen *Candida albicans* is an important cause of superficial mucosal infection and systemic disease in humans (15). Chronic hyperplastic candidosis (CHC) is a form of oral candidosis characterised by hyphal invasion of the oral epithelium (5, 6) and is clinically significant because of its reported association with squamous cell carcinoma (SCC). The development of malignancy at CHC lesional sites has been estimated to occur in up to 10% of untreated cases (2, 6, 7). Candidal virulence attributes involved in SCC development are unknown and still a matter of debate, although production of endogenous nitrosamines by strains of *C. albicans* has been implicated (13). Factors involved in the occurrence of CHC are also unclear, although host factors including tobacco smoking, denture wearing, and regular alcohol intake are believed important (1). Such host factors are also implicated in other forms of oral candidosis, particularly chronic erythematous candidosis and pseudomembranous candidosis (3). Consequently, it may be possible that *Candida* strains involved in CHC possess specific virulence attributes that promote CHC over other forms of oral candidosis. Since hyphal invasion of the stratum corneum is a distinguishing feature of CHC (5), our hypothesis is that expression of virulence factors involved in tissue invasion is significant for CHC isolates and may be less readily expressed by non-CHC strains. Previous studies have reported the importance of hydrolytic enzymes, such as secreted aspartyl proteinases (SAPs) (11, 19) and phospholipases (8) in candidal tissue invasion, whilst more recently, expression of agglutinin-like sequence genes has received attention due to their involvement in candidal adhesion (9, 10). SAP gene expression has been examined in a commercially available reconstituted human oral epithelium (RHE) to assess the role of these genes in tissue invasion (20). These experiments have showed temporal expression of SAP genes (SAP 1, SAP 3, SAP 6, SAP 8, and SAP 2) for *C. albicans* SC5314. The aim of the present study was to utilise the RHE culture model to assess the invasive capability of *C. albicans* isolated from CHC and other oral mucosal conditions. By comparing depth and pattern of tissue invasion by isolates, the relative 'virulence' of CHC strains in the infection model, compared with isolates not associated with invasive infection could be ascertained.

Materials and methods

Isolation and culture of test isolates

Nineteen isolates of *C. albicans* from four distinct clinical conditions (normal oral mucosa, $n = 4$; non-CHC oral candidoses, $n = 4$; CHC, $n = 7$; SCC, $n = 4$) were included in the study. Isolates were obtained from patients by imprint culture, where defined lesions were evident, and concentrate rinse culture (14, 17) for non-defined infection sites. Identification of *Candida* was based on germ-tube formation and the API 20C system (bioMérieux, Basingstoke, UK; 22).

Isolates were cultured overnight in 10 ml of yeast nitrogen base medium (YNB: Becton Dickinson Microbiology; UK supplier Fahrenheit Scientific, Rotherham, UK) supplemented with 0.5% glucose, under gentle agitation at 37°C. Cultures were washed (× 3) in phosphate buffered saline (PBS; Sigma, Poole, UK) and the yeast enumerated using an improved Neubauer haemocytometer (Hawksley, London, UK). The yeast suspension was adjusted to a concentration of 4×10^7 yeast/ml and this preparation was used as the inoculum. Viable counts of each inoculum were also obtained using a spiral plater system (Don Whitley Scientific, Shipley, UK).

Invasion model

The reconstituted human oral epithelium used for the *in vitro* model was obtained from SkinEthic Laboratory (Nice, France) (Fig. 1). Human keratinocytes derived from a SCC of the buccal mucosa were cultured on a 0.5-cm^2 insert. Airlifted cultures were prepared in serum-free conditions for 5 days in a defined medium containing insulin (5 μg/ml) and devoid of antibiotics and antimycotics. A 50-μl portion of the standardised yeast suspension was placed directly onto the insert, which was then incubated for 24 h at 37°C in a humidified atmosphere enriched with 5% CO_2. A non-infected PBS control was included for comparison and in all cases the experiments were repeated on two separate occasions.

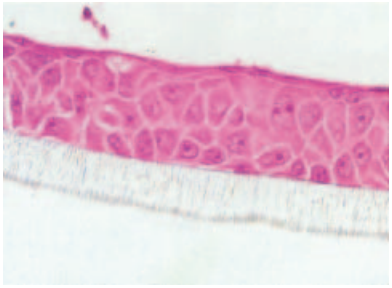

Fig. 1. Light micrograph of uninfected control tissue of reconstituted human oral mucosa stained with Haematoxylin & Eosin. Original magnification × 600.

Histological processing

After 24-h incubation, the tissue insert was removed and subjected to routine histological processing. The tissues were fixed in 10% formalin for 24 h prior to embedding in paraffin wax. Consecutive sections (5 μl) of the tissues were then stained with haematoxylin and eosin (to evaluate histological changes of the oral mucosa) and by the Periodic Acid Schiff method (to detect invading candidal hyphae). The characteristics of candidal invasion were subsequently recorded as no invasion, localised invasion or uniform invasion depending on the depth of hyphal penetration through the epithelium, extent of damage to the tissue, and the proportion of hyphae and yeast on the insert.

Results

Tissue invasion

Hyphal invasion of the epithelium was evident with all but one isolate (40/01) of the 19 *C. albicans* isolates examined (Table 1). The extent of invasion was isolate-dependent and consistent for a given isolate in repeat experiments. Overall, the ability to invade appeared to be correlated with the isolates' capacity to exhibit yeast to hyphal transition. The typical depth of tissue invasion varied between two and four epithelial cell layers (Table 1). When the isolates were grouped according to their clinical origin, there was no obvious association between CHC and the ability to invade the oral epithelium. This was typified by the finding that amongst CHC isolates, extensive invasion

Table 1. Pattern of invasion of reconstituted human epithelium by 19 isolates of *C. albicans*

Isolate reference	Origin	% Hyphae[a]	Cell depth of invasion[a]	Pattern of invasion[a]	Additional observations
DW1/93	normal oral mucosa	10%	2	localised	Superficial infection with some foci of invasion
PB1/93	normal oral mucosa	50–80%	4	uniform	Extensive cell damage
WK1/93	normal oral mucosa	90%	3–4	localised	Foci of invasion, cell damage
LR1/93	normal oral mucosa	5–25%	2	localised	Surface yeast prevalent with areas of deeper invasion
408/99	SCC, tongue	10%	2–3	localised	Foci of yeast infection, variation in cell and nuclear size
135BM2/94	CHC-SCC, buccal mucosa	100%	4	localised	Predominance of hyphae and tissue invasion
289T/00	SCC, tongue	10%	4	localised	Foci of invasion, cell damage
480/00	SCC, oral mucosa	20%	3	uniform	Superficial infection with foci of invasion, cell damage
819/99	keratosis, sublingual	25%	2	localised	Surface yeast/hyphae prevalent with limited invasion, areas of normal and thinned epithelium
970/00	CAC, oral mucosa	20–50%	2	uniform	Thinned epithelium and deeper cell damage
40/01	PMC, palate	NA	0	no invasion	Cell damage evident
243/00	lichen planus	25–40%	2	localised	Largely superficial infection with cell damage
PTR/94	CHC, buccal mucosa	80–100%	3–4	uniform	Surface penetration, thinned epithelium, pseudomembranous appearance
705/93	CHC, buccal mucosa	20–75%	2–3	localised	Yeast-like infection inducing cell damage
848/99	CHC, tongue	20–50%	2–3	uniform	Pseudomembranous-like infection with thinned epithelium
458R/94	CHC, buccal mucosa	50%	2–3	uniform	Pseudomembranous-like infection
324LA/94	CHC, commissure	100%	4–5	localised	Hyphal invasion through to membrane
455rgh/94	CHC, tongue	50%	2	localised	Yeast-like infection, surface epithelium, focal invasion
1190/97	CHC, buccal mucosa	10–20%	3–4	localised	Yeast-like infection, surface epithelium, focal invasion
PBS control	4–6 cell depth	NA	NA	NA	

SCC, squamous cell carcinoma; CHC, chronic hyperplastic candidosis; CAC, chronic atrophic candidosis; PMC, pseudomembranous candidosis. PBS, phosphate buffered saline.
[a]Replicate results; NA, not applicable.

occurred with strain 324LA/94, whilst poor invasion was noted with strain 455rgh/94. Furthermore, strains PB1/93 and WK1/93 which were classed as commensal isolates, demonstrated a relatively high degree of invasion.

The actual patterns of invasion varied between isolates (Table 1). Invasion patterns were based on the distribution of candidal elements within the tissue after 24 h of infection. In this respect, two distinct forms of invasion were noted and categorised as either uniform or localised (Figs 2 and 3). Uniform invasion was characterised by a regular distribution of hyphae or yeast throughout the cell layers. In contrast, localised invasion was evident when foci of invading hyphae within distinct areas of the epithelium were detected. In several cases, large numbers of yeast and hyphae predominated at the surface of the oral mucosa with limited penetration, a pattern of infection not dissimilar to that reported for pseudomembranous candidosis (16) (Fig. 4).

Extensive penetration of the mucosa appeared to correlate with increased numbers of hyphal elements, especially when invasion of the lower cell layers occurred. Sub-epithelial invasion tended to result in the detachment of the epithelium from the membrane base, accompanied by a hyphal to yeast transition.

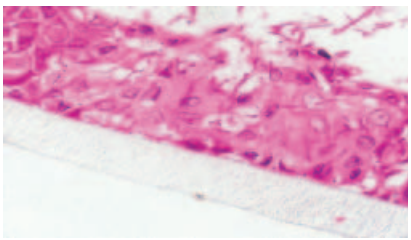

Fig. 2. Uniform invasion by *C. albicans* in the oral epithelium. Stained with Haematoxylin & Eosin. Original magnification × 600.

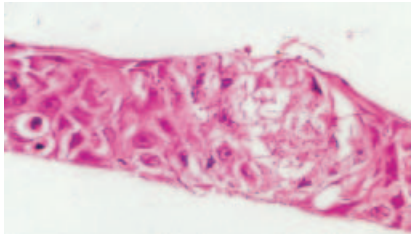

Fig. 3. Focal hyphal invasion of the oral epithelium with strong oedema and vacuolisation. Stained with Haematoxylin & Eosin. Original magnification × 600.

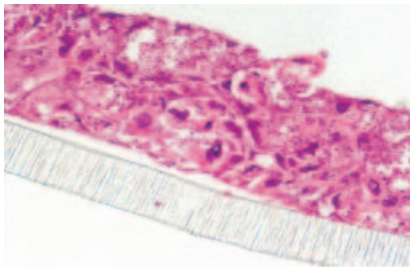

Fig. 4. Diffuse yeast infection of the oral epithelium with limited hyphal invasion. Stained with Haematoxylin & Eosin. Original magnification × 600.

Discussion

One particular pathogenic feature of *Candida* that would appear essential in the development of CHC is its ability to invade oral mucosa. Indeed, one of the primary features distinguishing CHC from other forms of candidal infection is the presence of *Candida* hyphae within the epithelium. In the present study, an *in vitro* tissue model that had been used previously to examine candidal hydrolytic enzyme gene expression (12, 13, 18) was employed to compare the invasive capabilities of isolates of *C. albicans*.

The present study confirms the suitability of the tissue as an *in vitro* model for studying CHC. Features of chronic mucosal infection were observed, as was the invasion of candidal hyphae. The obvious drawback of the system was that it lacked the intrinsic immune response of normal oral mucosa. Nevertheless, the oral mucosal model did prove successful in highlighting consistent differences in the ability of strains of *C. albicans* to invade, both in terms of depth and pattern of invasion. These differences probably relate, in part, to variations in a specific isolate's ability to develop filamentous forms, multiply within the tissue, or produce tissue-degrading hydrolytic enzymes. Our finding of distinct patterns of invasion (focal and uniform) is in agreement with a previous study (19) using *C. albicans* SC5314, where regular or focal invasion of the epidermis was evident after 24 h infection.

Overall, the findings reported here do not suggest a relationship between the clinical source of the isolate and the ability to invade the tissue model. This is highlighted by the extremes of invasion exhibited by those isolates originally obtained from normal oral mucosa (commensal isolates). However, it is worth noting that the four isolates from SCC were consistently high level invaders and further work is required, using a larger number of isolates from SCC, to establish whether this result is significant. Previous investigations using a similar reconstituted oesophageal tissue model reported tissue invasion by all clinical isolates examined, but not by commensal or reference collection strains (4). Notwithstanding the relatively small numbers of isolates used in this study, our inability to identify a heightened level of invasion specifically for CHC isolates suggests that either other isolates (exhibiting high levels of invasion) are capable of causing CHC or that candidal invasion is not singly responsible for CHC. The former is indeed likely since it is widely recognised that host factors are also significant in determining whether oral candidosis occurs. Isolates in this study have been categorised as being 'commensal', due to the nature of the host from where they were recovered as opposed to the inherent virulence of the organism. If invasion alone is not responsible for inducing the histological features of CHC, then the production of specific virulence factors such as hydrolytic enzymes or the expression of specific antigenic properties could be important. Schaller et al. (18) demonstrated the suitability of the mucosal model for examining candidal SAP expression. This could be further extended using techniques such as differential display to characterise gene expression during the invasion process. However, until such studies are performed, current evidence would seem to indicate that whilst heterogeneity exists with regard to candidal virulence attributes, host factors are still principal elements in the progression of CHC infection.

Acknowledgments

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