The relationship between colonisation, secretor status and in-vitro adhesion of *Candida albicans* to buccal epithelial cells from diabetics

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Summary. This study investigated whether oral candida infection in diabetics and adhesion of *Candida albicans* to buccal epithelial cells *in vitro* were related. Buccal cells from 50 patients with diabetes mellitus showed a significant increase in adhesion of *C. albicans* strain CDS 88 compared with those collected from 50 non-diabetic controls matched for age, sex and denture status. Oral candida carriage, candida infection and secretor status were also investigated in both groups. The frequency of carriage was increased, but not significantly, and there was a significantly higher incidence of candida infection in diabetic patients compared with controls. Diabetic patients who were non-secretors had a significantly increased frequency of oral candida carriage.

Introduction

The adhesion of micro-organisms to host mucosal surfaces is a necessary pre-requisite for successful microbial colonisation and infection,[1] and the role of adhesion in the pathogenesis of many fungal infections is widely appreciated.[2] Various animal studies have provided evidence for a relationship between the propensity of *Candida* species to adhere to mucosal surfaces and their presence in infections.[3, 4]

There are quantitative and qualitative differences in oral candida colonisation in patients with diabetes mellitus. In some studies, the oral carriage rate of candida has been estimated to be as high as 80%[5] and others have also reported an increase in the number of yeasts in such patients.[6, 7] We have reported previously an increased prevalence of oral candida infections in diabetic patients compared with closely matched control subjects.[8] In an attempt to explain this increased prevalence, factors which may have contributed to this phenomenon such as control of glycaemia and type and duration of metabolic disease have been studied, but no relationship could be established with either oral candida carriage or infection.[8–10] However, the genetically determined inability to secrete the water-soluble glycoprotein forms of the ABO blood group antigens in saliva has been described in association with increased incidence of oral candida carriage[11] and infection.[12] The predisposition of diabetic patients to cutaneous[13] and vaginal candidosis[14] has been recognised but not fully explained, although the increased predisposition of female patients with diabetes mellitus to vaginal candidosis has been explained partly by increased candidal adhesion to their vaginal epithelial cells *in vitro*.[15] In this study, the adhesion of *C. albicans* to buccal epithelial cells from patients with diabetes mellitus and non-diabetic control subjects was compared and the effects of secretor status, control of glycaemia and haematinic status on this were assessed.

Materials and methods

Fifty patients with diabetes mellitus were randomly selected from an out-patient Diabetic Clinic at Glasgow Royal Infirmary. The control group comprised 50 non-diabetics matched for age, sex and denture status, recruited either from those attending the Oral Medicine Unit migraine clinic for the first time or from patients attending the Department of Prosthodontics, Glasgow Dental Hospital for denture review. None of the control subjects was attending for treatment of any oral disease. Smoking and alcohol drinking habits were closely matched between individuals of both groups and no individual in either group had received antibiotic or steroid therapy or used an antiseptic mouth wash for at

least 1 month before entering the study. All participants had a full blood count, corrected whole-blood folate and ferritin, vitamin B_{12}, haemoglobin and random blood glucose estimations. Each diabetic patient also had a blood sample assayed for glycated haemoglobin. The quality of diabetic control was designated as "good" when the glycated haemoglobin level was $< 10\%$, "fair" when $10–12\%$ and "poor" when $> 12\%$. This somewhat arbitrary designation is widely accepted clinically.

The mouths of each diabetic patient and control subject were examined and the presence of angular cheilitis or oral candida infection recorded.

Microbiological investigations

Swabs and smears were collected from areas of mucosa that appeared infected and from the fitting surface of the upper denture in cases of chronic atrophic candidosis. Smears were air dried and stained by Gram's method. Swabs were inoculated on to Sabouraud's Dextrose Agar (Gibco). Oral yeast colonisation was assessed by an oral rinse technique described by Samaranayake et al.[16] For this, each subject was asked to rinse the mouth thoroughly, after removal of dentures if appropriate, with 10 ml of sterile phosphate-buffered saline (PBS; 0·1 M, pH 7·2) for 1 min. The rinse was centrifuged at 1700 g for 15 min and the deposits were reconstituted in 1 ml of PBS. A spiral plater (model D; Spiral System Marketing Ltd, MD, USA) was used to dispense 50 μl of the deposit on to Sabouraud's Dextrose Agar (Gibco). All cultures were incubated aerobically at 37°C for 48 h.

C. albicans and other Candida spp. were identified by germ-tube formation[17] and by API 20 C AUX (API Products Ltd, Basingstoke, Hants).

On the basis of candida isolation and signs of oral candidosis, the subjects were subdivided into yeast-free, asymptomatic yeast carriers, and candidosis groups.

Determination of secretor status

Whole unstimulated saliva (2 ml) was collected from each subject and placed on ice for determination of secretor status by the haemagglutination inhibition technique of Periera et al.[18] Individuals whose saliva did not inhibit blood-cell agglutination or did so at a titre of $\leqslant 16$ were designated non-secretors.[18]

Candida adhesion assay

The C. albicans strain (CDS 88) used in the adhesion assay had been isolated from the mouth of an asymptomatic carrier, identified as described above and then freeze dried. During experiments, the strain was maintained on slopes of Sabouraud's agar, at 4°C. The maintenance culture was replaced every month with freshly grown organisms from a freeze-dried culture. A loopful of the stock culture was inoculated into 10 ml of Sabouraud's Dextrose Broth (Gibco) containing 500 mM sucrose and incubated for 18–24 h at 37°C in an orbital

shaker at 100 rpm. The culture was harvested by centrifugation at 400 g for 10 min and the deposit was washed twice, each time with 10 ml of PBS. A final suspension of 10^7 yeasts/ml was prepared by dilution of this deposit in PBS and the concentration was checked by counting in a haemocytometer.

Buccal epithelial cells (BEC) were obtained from each subject by gently rubbing the right and left buccal mucosa with sterile cotton swabs. BEC from each subject were pooled by agitating the swabs in 10 ml of PBS in a universal container. The cells were washed twice in 10 ml of PBS to remove loosely attached micro-organisms and centrifuged at 200 g for 10 min. The BEC were then suspended in PBS at a concentration of 10^5 cells/ml.

For the adhesion assay, 0·5 ml each of BEC and C. albicans suspensions were mixed in a sterile 7-ml screw-capped glass container and incubated in an orbital shaker at 80 rpm at 37°C for 1 h. Each experiment had a control that contained 0·5 ml of BEC suspension and 0·5 ml of PBS. The cells were then harvested on 12 μm-pore polycarbonate filter (Millipore, Gmbh, Germany) and washed with 30 ml of PBS in six 5-ml volumes to remove unattached yeasts. The filter was then placed on a glass microscope slide, air dried and stained by Gram's method.

C. albicans blastospores attached to 100 randomly-selected BEC were counted by microscopy at a magnification $\times 40$. Only single BEC were counted and clumps of cells were excluded. The background control count, if present, was subtracted from the total count.

Statistical analysis

The proportions of those with yeasts and the proportions of secretors in each group were compared by the χ^2 test. Values for in-vitro candida adhesion in both groups were accepted as being normally distributed and compared by Student's t test. Since the numbers of cfu/ml obtained from subjects of both groups were not normally distributed, a Mann Whitney U test was used to compare the quantitative candida isolation from each group and the median and the interquartile range (Q1, Q3) were used to present data of quantity and spread of candida isolation. Pearson's correlation coefficient was used to measure the associations between oral candida carriage, in-vitro adhesion, and the other parameters examined. In all tests, a p value $< 0·05$ was considered significant.

Results

The diabetic patients and non-diabetic subjects were matched for age, sex and dental status (table I). The diabetic group comprised 19 insulin-dependent and 31 non-insulin-dependent diabetics. The mean duration of disease was 6·7 (SD 6·9) years with a range of 4 months to 32 years. The quality of control of glycaemia was "good" in 25 (59·5%), "fair" in 13 (27%) and "poor" in only 4 (9·5%) patients. Blood glycated haemoglobin esti-

Table I. Sex, age and denture status of diabetic patients and controls

| Group (n) | Male | Female | Mean (SD) age (years) | Denture status | | |
				CD	PD	Dentate
Diabetics (50)	27 (54%)	23 (46%)	53·7 (14·5)	24 (48%)	12 (24%)	14 (28%)
Controls (50)	23 (46%)	27 (54%)	56·3 (17·3)	26 (52%)	10 (20%)	14 (28%)

CD, complete dentures; PD, partial dentures.

mation was technically unsatisfactory in eight diabetic patients.

Candida spp. were isolated from the mouths of 27 (54%) of the diabetic patients (median cfu/ml 1140; Q1, Q3 = 160, 3300) and from 20 (40%) of non-diabetics (median of cfu/ml 310; Q1, Q3 = 120, 1950) but the differences between the two groups were not significant. Fisher's exact probability test showed that diabetic patients had a significantly increased incidence of oral candida infection; six (12%) of the diabetic patients had clinical and microbiological evidence of oral candida infection (median of cfu/ml 8000; Q1, Q3 = 2725, 36176) whereas the controls had none (p < 0·02). *C. albicans* comprised 77·7% and 75% of the yeast isolates in diabetics and controls respectively, and *C. glabrata* 15% in both groups.

There were no significant relationships between frequency or quantity of candida isolation and age, sex or duration and type of diabetes mellitus, and there were no significant correlations between blood glucose concentration, glycated haemoglobin, corrected whole-blood folate, ferritin or vitamin B_{12} and quantity of candidal isolation in either group. The correlation overall between quality of diabetic control and frequency or quantity of candida isolation was not significant but numbers of candida were significantly higher in patients with "fair" than in patients with "good" diabetic control (p < 0·05) (table II).

Neither the frequency nor quantity of candida isolation was significantly different between dentate individuals and denture wearers in either group. In diabetic patients, *Candida* spp. were isolated from 22 (61%) denture wearers (median cfu/ml 650; Q1, Q3 = 165, 2015) and five (36%) dentate patients (median cfu/ml 140; Q1, Q3 = 120, 4140). In the controls, *Candida* spp. were isolated from 16 (44%) denture wearers (median cfu/ml 280; Q1, Q3 = 120, 11040) and four (29%) dentate control subjects (median cfu/ml 890; Q1, Q3 = 155, 1820). However, in diabetic patients who wore dentures continuously there was a significant increase in the frequency of candida isolation compared with those who wore dentures only during the day (92% cf 43%; p < 0·01).

Analysis of saliva showed that there were similar numbers of diabetic and non-diabetic individuals who were secretors of blood group substances. Non-secretor diabetic patients had a significantly increased frequency of candida isolation (p < 0·05) (table III).

The mean candida adhesion (MCA) of *C. albicans* strain CDS 88 to BEC from diabetic patients was 4·02 (SD 1·85) yeasts/BEC and 2·57 (SD 1·2) yeasts/BEC for control subjects (figure). The difference between the two groups was highly significant (p < 0·001). The mean number of BEC with one or more adherent blastospores (candida-positive cells) and the number with no adherent blastospores (candida-negative cells) were calculated for each individual (100 BEC from each person were counted). The mean number of candida-positive cells was 63 (SD 11) in diabetic patients and 54 (SD 14) in the control

Table II. Relationship of diabetic control to candida isolation and in-vitro adhesion

Diabetic control (n)	Frequency of candida isolation	Median cfu/ml (Q1, Q3)	Candida adhesion Mean (SD) yeast cells/BEC
Good (25)	13 (54%)	180 (100, 500)	2·8 (1·4)
Fair (13)	6 (46%)	2070 (885, 3225)	3·9 (1·7)
Poor (4)	3 (75%)	1460 (140, 5280)	3·6 (3·5)

Table III. Relationship of secretor status to isolation of yeast and candida infection

Group (n)	Yeast isolation*	Species	Infected	Species	Yeast-free
Diabetics					
Secretors (31)	13 (42%)	13 C. albicans	3 (10%)	3 C. albicans	18 (58%)
Non-secretors (17)	13 (76%)	10 C. albicans	3 (18%)	1 C. albicans	7 (41%)
		2 C. glabrata		1 C. glabrata	
		1 C. krusei		1 C. krusei	
Controls					
Secretors (31)	10 (32%)	7 C. albicans	0		21 (68%)
		3 C. glabrata			
Non-secretors (19)	10 (53%)	7 C. albicans	0		9 (47%)
		1 C. glabrata			
		1 C. stellatoidea			
		1 C. pseudotropicalis			

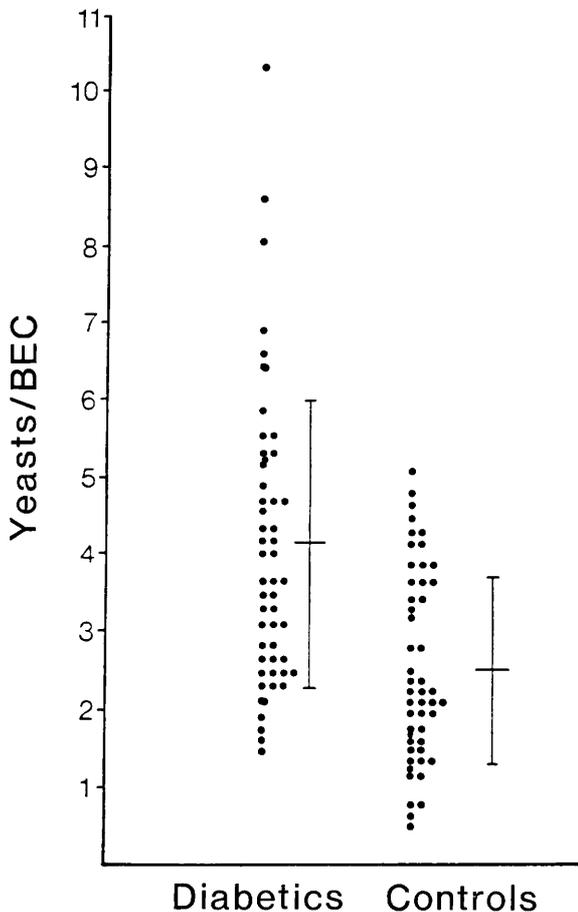* Number of patients with oral candida infection included.

Figure. Candida adhesion to BEC from diabetic patients (mean 4·02 SD 1·85) and controls (mean 2·57 SD 1·2).

group. The difference between the two groups was highly significant ($p < 0.001$). The type of diabetes did not affect candida adhesion; MCA was 4·1 (SD 1·98) for insulin-dependent diabetics and 3·9 (SD 1·7) for non-insulin-dependent diabetics (not significant).

There was a negative correlation between the MCA in diabetic patients and the overall quality of diabetic control, but the differences between the groups were not statistically significant (table II). Furthermore, linear regression analysis showed no significant correlation between MCA and blood glucose, glycated haemoglobin, folate, ferritin or vitamin B_{12} levels. In control subjects there was a significant negative correlation ($p < 0.05$) between MCA and haemoglobin concentration, but this was not significant in diabetic patients. In the control group, but not in the diabetic group, females had significantly greater MCAs than males ($p < 0.02$; table IV).

Although no significant relationship was detected between MCA and denture status generally, control subjects who wore their dentures continuously had significantly higher MCA values than those who wore dentures only during the day ($p < 0.02$; table IV).

Diabetic patients and control subjects were each divided into subgroups according to oral candida isolation. There was no significant difference in MCA between diabetic patients who harboured yeasts (3·9 SD 1·9) or were yeast-free (4·1 SD 1·9) or between colonised and yeast-free controls. Diabetic patients with candida infection did not have higher MCA (3·2 SD 1·0) than asymptomatic candida carriers (4·1 SD 2·0) or yeast-free patients (4·1 SD 1·8). There was no correlation between

Table IV. Relationship of candida adhesion to sex, dental status and denture wearing patterns

Group	Candida adhesion, mean (SD) yeast cells/BEC								
	Male	vs	Female	Dentate	vs	Denture	Denture wearing		
							D&N	vs	D
Diabetics	4·2 (1·8)	–NS–	3·8 (1·9)	4·7 (2·0)	–NS–	3·7 (1·7)	3·5 (1·0)	–NS–	3·7 (1·0)
Controls	2·1 (1·1)	–p<0·02–	3·0 (1·2)	2·7 (1·5)	–NS–	2·4 (1·1)	3·1 (1·1)	–p<0·02–	2·2 (1·0)

NS, not significant.
D&N, continuous denture wearing (day and night); D, denture wearing (day only).

MCA and candida counts in individuals who harboured yeasts in either group.

In diabetic patients the MCA was higher in non-secretors (4·5 SD 1·9) than in secretors (3·7 SD 1·8) but the difference was not significant. Similarly, in the control group the difference in MCA between secretors and non-secretors (2·5 SD 1·1 cf 2·6 SD 1·3) was not significant.

Discussion

To find that 54% of diabetic patients harboured *Candida* sp. in the mouth is consistent with a report of previous studies,[8] and confirms the view that oral candida isolation is more frequent in diabetic patients than in non-diabetics.[5, 6, 9] Furthermore, the presence of oral candida infection in 12% of diabetic patients but in none of the closely matched controls supports the view that oral candida infections are more common in diabetic patients than in non-diabetics. The results of this study also confirm our previous observation that there is no significant difference in the quantity of oral candida colonisation between diabetic and non-diabetic individuals.[8] These results are contrary to the finding of Tapper-Jones *et al.*[6] who reported higher candida counts in diabetics.

C. albicans adheres to buccal and vaginal epithelial cells *in vitro* to a greater degree than other *Candida* spp.[19] and this may explain why some species are isolated more frequently from mucosal surfaces. The increased susceptibility of diabetic women to vaginal candidosis has been linked with increased adhesion of *C. albicans in vitro* to vaginal cells collected from these patients.[15] In this study we found that the adhesion of *C. albicans* to BEC of diabetic patients was significantly greater than to BEC from non-diabetics. This increased adhesion was observed even when diabetics and non-diabetics had similar levels of candida carriage.

The lack of correlation between candida carriage and candida adhesion *in vitro* implies that factors other than adhesion e.g., the presence of dental prostheses, denture wearing patterns[20] or high carbohydrate intake,[21] may promote carriage of *Candida* spp. in the oral cavity. The failure to demonstrate a difference in adhesion between yeast carriers and yeast-free individuals in this study is in contrast to the results of Cox[22] who found significantly increased candida adhesion to epithelial cells from colonised subjects and from patients with oral candidosis compared with cells from controls. However, 95% of the colonised and 80% of the infected subjects in his study were receiving antibiotic therapy at the time of testing and this might explain the increased candida adhesion.[23] Antibiotic therapy supresses indigenous oral bacteria competing with yeasts for nutrients[24] and adhesion sites on epithelial cells.[25]

The greater adhesion to BEC of diabetic patients may account for the increased prevalence of candida infection since adhesion to epithelial cells is essential for consequent invasion and infection.[26] Adhesion of *C. albicans* to epithelial cells is generally the result of interaction between candida adhesins and complementary receptors on epithelial cells,[2] which are glycosides containing a protein and a carbohydrate moiety.[27] Since all adhesion assays in this study were done with the same conditions and with a single *C. albicans* strain, the differences in adhesion to BEC from diabetics and non-diabetics may reflect true differences in the number or nature of the available receptors on the BEC surface.

Long standing diabetes is frequently associated with permanent and irreversible functional and structural changes in many cells throughout the body.[28] Glucose in blood forms glycosylation products with tissue proteins at a rate porportional to the glucose concentration. Hence the amounts of glycosylation products of haemoglobin, or other proteins in basement membranes or endothelial cells, are much increased in diabetic patients.[29]

Therefore, it would be interesting to investigate the possibility that alterations in the glycation process in diabetic patients may affect carbohydrate moieties on the epithelial cells in a way that might modify their receptivity to yeast cells. A phenomenon such as this may account for the significant increase in candida-positive cells observed in diabetics. However, the increased candida adhesion in diabetic patients did not correlate with concentrations of blood glucose or glycated haemoglobin at the time of sampling. Nevertheless, Segal *et al.*[15] concluded that increased candida adhesion to vaginal epithelial cells from diabetic women was related to the increased glycogen content of these cells and to the higher proportion of epithelial cells with larger surface areas.

The high prevalence of oral candida carriage associated with denture wearing in both groups of this study, particularly in diabetics who wear dentures continuously, is consistent with that reported among denture wearers generally.[20] *C. albicans* adheres to acrylic,[30] therefore dentures may act as an additional reservoir for these organisms. However, these factors do not explain the significantly increased candida adhesion to buccal cells of "continuous" denture wearers compared with control subjects as found in this study.

The negative correlation between haemoglobin level and in-vitro candida adhesion in the non-diabetics cannot be explained; nevertheless, it may be relevant to the reported increased incidence of oral candida infection in patients with iron deficiency.[31, 32]

In this study, similar proportions of diabetics and non-diabetics secreted blood group substances in saliva. The non-secretors had a higher prevalence or oral candida carriage but this was only significant in diabetic patients. These blood group substances may protect the host by occupying or interfering with binding sites, either on the surface of microorganisms or on epithelial cells, thus adversely affecting colonisation and invasion.[33] May *et al.*[12] reported that epithelial cells from secretors bound fewer candida cells than those from non-secretors. Our results tend to support the latter findings, which suggests that non-secretor diabetic patients may have an increased risk of developing oral candidosis, due to increased likelihood of harbouring yeasts in the mouth and increased tendency of the BEC to become colonised. Whether the increased affinity of the BEC of diabetics for candida is an intrinsic property mediated by the metabolic disorder remains to be determined.

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