Susceptibility of *Candida albicans* isolates from oral cavities of HIV positive patients to Histatin-5.

Abstract

Statement of problem. There is a possibility that the colonization of oral surfaces, including the denture fitting surface, can serve as a reservoir for disseminated Candidal infections, particularly in immuno-compromised hosts, such as AIDS patients. Histatins are a group of small, cationic antifungal peptides present in human saliva. Several recent reports have suggested the therapeutic potential of this peptides in patients with oral candidosis. However, little information is available on the antifungal activity of the peptides agaist *Candida albicans* isolates from HIV positive patients.

Purpose. Fungicidal effects of Histatin-5 against oral isolates of *Candida albicans* from HIV-positive and -negative patients were examined.

Methods/Materials. Two isolates of *C. albicans* from HIV-positive patients (two males), three isolates from HIV-negative patients (two males amd one female healthy donors) and one ATCC strain were used. Fungicidal assays were performed on exponential *C. albicans* cells in the presence or absence of Histatin-5 (0.315-50 mM).

Results. Fifty mM Histatin-5 killed more than 95% of *C. albicans* isolates from HIV-negative patients and killed more than 90% of ATCC strain. In contrast, Histatin-5 induced 75.3% and 66.1% loss of viability of two *C. albicans* isolates from HIV-positive patients, respectively, which were statistically less effective than the fungicidal effects against any isolates from HIV negative patients, or a reference strain (ANOVA; $p<0.05$).

Conclusion. *C. albicans* isolates from oral cavity of HIV-positive patients were less sensitive to Histatin-5, as compared with the oral isolates from HIV-negative patients.

Clinical Implications Although our results demonstrated the therapeutic potential of Histatin-5 against oral candidosis, we pointed out that the antifungal effects of the peptide against isolates of *C. albicans* from HIV-positive patients were relatively low. Thus the improvement of efficacy of the peptides should be required.

The possibility that the colonization of oral surfaces, including the denture fitting surface, can serve as a reservoir for disseminated infections, such as aspiration pneumonia and gastrointestinal infection have been pointed out.[1] There are indeed, reports indicating that pleuropulmonary infections may arise from aspiration of oral flora including yeasts,[2,3] and a case of aspiration candida pneumonia in a non-immunosuppressed host has also been reported.[4] Although it is known that candida pneumonia in immunocompetent adults is very rare, it should be noted that there are increasing risks of such infection in elderly patients who are receiving antibiotic therapy, radiation or immunosuppressive medications,[5] or in immuno-compromised host, such as AIDS patients.

The AIDS epidemic, improved life-sustaining therapy, and aggressive anticancer therapy have contributed to the rise in the number of severly immunocompromised patients.[6] This has led to an increase in oral and systemic fungal infection, and the concominant increased usage of antifungal agents for prophylaxis is most likely the main cause of the development of antifungal drug resistance.[7] Particularly, Barchiesi et al. (1995) have suggested that *C. albicans* isolates from oral cavities of HIV positive patients will be less susceptible in vivo to standard doses of fluconazole.[8] In addition, unfortunately, most of currently available antifungal drugs, such as polyene and azole antimycotics, have undesirable toxic and other side effects.[9] Therefore, the search for more effective but less toxic anticandidal theurapeutic agents cannot be overemphsized. Peptide antibiotics, which are believed to interact with the microbial membrane leading to the disruption of cellular integrity and cell death, may be a promising new class of antifungal agents.[6]

Histatins are a group of small, cationic antifungal peptides present in human saliva.[10,11] They also have been detected in human serum.[12] In saliva, secretory IgA, lactoferrin[13-15], lysozyme[16,17], and histatins[11] have all been demonstrated to exert anticandidal activities. Among these molecules, histatins are most likely to be the proteins responsible for in vitro salivary candicidal activity.[18] Since histatins are naturally occuring molecules they are considered to be nontoxic to mammalian cells[21]. This property and the fungicidal potencies make histatins promising natural therapueic or preventive agents against fungal infection. Among the three major histatins (histatin-1, -3 and -5), Histatin-5 is the most potent peptide in killing *Candida albicans* isolates[22].

Histatin-5 has been considered to play one of the most important role in primary (salivary) protection against oral Candidosis, particularly in immunocompromised host. In fact, amano several protective proteins in saliva, only the histatin concentration demonstrates a statistically significant decrease in the saliva of AIDS patients who developed candidiasis[19]. The decrease in histatin concentration in the saliva of AIDS patients[18] may partially explain

why more than 70% of AIDS patients develop oral candidiasis over the course of the disease[20]. Thus, so far, the decrease in the Histatin concentration of saliva has been thought to be of importance in the development of Candidosis in AIDS patients. However, little is known about the susceptibility of *Candida albicans* isolates from HIV positive patients to this peptide. Thus, in the present study, we analysed the fungicidal potential of Histatin-5 against 2 isolates of *C. albicans* from oral cavities of HIV positive patients.

MATERIALS and METHODS

Candida albicans, growth conditions and agent

C. albicans A1, A2, A6, A8, A10 and ATCC 90028 (American Type Culture Collection, Manassas, VA, USA) were kindly provided from Prof. Samaranayake, Oral Biology Unit, Prince Phillip Dental Hospital, Hong Kong University, and used in the present study. *C. albicans* A1 and A2 were the oral isolates obtained from HIV-positive patients (2 males). Both of them are in early stage of AIDS, and the general status of them are healthy other than AIDS. *C. albicans* A6, A8 and A10 were the oral isolates from HIV-negative patients (healthy, 2 males and 1 female donors) isolated in Oral Biology Unit of Hong Kong University, China. *C. albicans* ATCC 90028 was used as the reference strain. All the isolates were identified by sugar assimilation tests using the API 20C system (API Products, Biomeroux, Lyon, France) and the "germ tube" test.[23]

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.[24] After 18 h incubation, the yeast was harvested in the mid exponential growth phase, washed twice with PBS containing 1 mM phosphate (pH 6.8) and resuspended to a final concentration using a haemocytometer.[17, 25] All yeast cells suspended, remained in the blastospore phase.

Histatin-5 (amino acid sequence; DSHEKRHHGYKRKFHEKHHSHRGY;IUPAC-IUB) was purchased from Sigma Chem. Co. (St Louis, MO, USA).

Candidacidal assay

Antifungal activity of Histatin-5 was examined according to the method of Edgerton et al. (1998) with some modifications.[26] Fungicidal assays were performed on exponential *C. albicans* cells in the presence or absence of Histatin-5 (0.315-50 mM). Briefly, *C. albicans* cells were washed twice with 1 mM sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4; pH6.8), and resuspended at 1.8×10^5 cells/ml. Twenty microliter of cell suspensions were mixed with

20 ml of 1 mM phosphate buffer containing the indicated protein and incubated for 90 min at 37 °C with shaking. Control tubes were incubated with 20 ml of 1mM phosphate buffer alone.

The reaction was stopped by addition of 360 ml of yeast nitrogen base (YNB); 40 ml of the suspension (containing 360 cells) were spread onto Sabouraud dextrose agar plates and incubated for 48 h at 37 °C.

The assays were performed on two independent occasions with quadruplicated specimens on each occasion. Loss of viability was calculated as [1 - (colonies from suspension with peptides/colonies from suspension with no protein)] x 100.[26]

Statistical analyses

All of the numerical data obtained here were analyzed by an one-way analysis of variance (ANOVA) and subjected to Tukey's multiple range test ($p<0.05$).

RESULTS

Since, in the preliminary study, fungicidal effect of Histatin-5 was gradually increased within 45-min incubation, and plateaued after 60-min incubation, we examined fungicidal effects of the peptide at 90-min incubation.

In the preliminary study, as compared with the control (0 mM), significantly increased fungicidal activity was observed with increasing concentration of Histatin-5 (Fig. 1). Thus in the further analysis, the fungicidal activity of Histatin-5 at the final concentration of 50 mM was employed. At this concentration, Histatin-5 killed more than 95% of *C. albicans* A6, A8 and A10 and killed more than 90% of ATCC strain (Fig. 2). In contrast, Histatin-5 induced 75.3% and 66.1% loss of viability of the *C. albicans* A1 and A2 cells, respectively (Fig. 2). Thus, *C. albicans* A1 was statistically less susceptible than any of oral isolates from HIV negative patients (Tables 1 and 2; $p<0.05$), while there was no significant differences between the sensitibity of *C. albicans* A1 and ATCC strain (Tables 1 and 2; $p>0.05$), which was used as a reference strain. In contrast, *C. albicans* A2 was statistically less sensitive than either isolates from HIV negative patients, and or a reference strain (Tables 1 and 2; $p<0.05$).

DISCUSSION

The family of salivary histatins consists of structurally related, low molecular weight histidine rich proteins, which are part of the non-immune host defense system of the oral-esophageal area.[26] Histatins are present in saliva of healthy adults at concentrations of approximately 50 - 425 mg/ml.[26] Histatin-5 is the most potent candicidal member of the family

that kills pathogenic *Candida* species from 90 to 100% at physiological concentrations.[27] The finding that histatins are potent *in vitro* antifungal agents, while nontoxic to human cells, provided promise for their therapeutic potential in patients with oral candidosis.

Sevaral studies have evaluated the Histatin-5 induced killing of various isolates of *C. albicans.* Edgerton et al. (1998) has examined the antifungal activities of histatins, including Histatin-5, and shown that concentration of Histatin-5 (31.25 mM) was required to kill more than 95% of *C. albicans* isolate derived from palate of denture stomatitis patients.[26] Thai and Bobek (1997) compared the fungicidal effect of recombinant Histatin-5 on one isolate of azole-sensitive with that on an azole-resistant isolate of *C. albicans*, which contains an increased level of sterol 14a-demethylase, and have shown that recombinant Histatin-5 killed both isolates more than 90% at the concentration of 50 mM.[21] Helmerhorst et al (1999) demonstrated 50 mM Histatin-5 killed more than 90% of the ergosterol-deficient mutant of *C. albicans* which was resistant to amphotericin B, and 100% of an amphotericin B sensitive isolate of *C. albicans.*[6] The findings, obtained here, that 50 mM of Histatin-5 induced more than 95% of loss of viability of all three isolates from oral cavities of HIV negative patients, and that killed more than 90% of ATCC strain, which was used as reference strain, were consistent with the results of previous studies. However, the same concentration of the peptide (which killed more than 95% of *C. albicans* isolates from HIV negative patients), induced only 75.3% and 66.1% loss of viability of the *C. albicans* A1 and A2 cells, isolated from oral cavity of HIV positive patients, respectively. The finding implies that the *C. albicans* isolates exsist in AIDS patients should be less susceptible to Histatin-5 in saliva, as compared with the isolates from HIV-negative patients. It has been considered that the decrease in histatin concentration in the saliva of AIDS patients[18] may partially explain why more than 70% of AIDS patients develop oral candidiasis over the course of the disease[20]. In addition to this, there is a possibility that the changes in susceptibility of *C. albicans* isolates to Histatin-5 may be attributed to the latter phenomena.

Although our results, from the limited number of *C. albicans* isolates, taken together, supported the therapeuic potentials of Histatin-5 for oral candidosis as previously suggested, we raised here the possible problem of application of Histatin-5 to the control of oral *Candida* isolates for HIV-positive patients. Further trials to improve the fungicidal potential of basic peptides should be required.

CONCLUSIONS

In the present study, we examined the fungicidal potential of Histatin-5 against totally 6 isolates of *C. albicans* (2 isolates from oral cavities of HIV positive patients, 3 isolates from oral cavity of HIV negative patients, and one from ATCC).

1. Increased fungicidal activity was observed with increasing concentration of Histatin-5 (0.315-50 mM).

2. Histatin-5 killed more than 95% of three isolates of *C. albicans* from HIV negative patients, and killed more than 90% of ATCC strain.

3. Histatin-5 induced 75.3% and 66.1% loss of viability of two isolates from HIV positive patients, which were statistically less effective than the fungicidal effects against either isolates from HIV negative patients.

Although our results, taken together, supported the therapeuic potentials of Histatin-5 for oral candidosis as previously suggested, there is the possibility that the decrease in susceptibility of *C. albicans* isolates to Histatin-5 may be attributed to the facillitation of Candidosis in AIDS patients.

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Legends to Figures

Fig. 1 Typical effects of concentration (0-50 mM) of histatin-5 on the loss of viability of *C. albicans* A8, fungal colonies and the changes in colony forming units are shown in figure, (a) and (b).

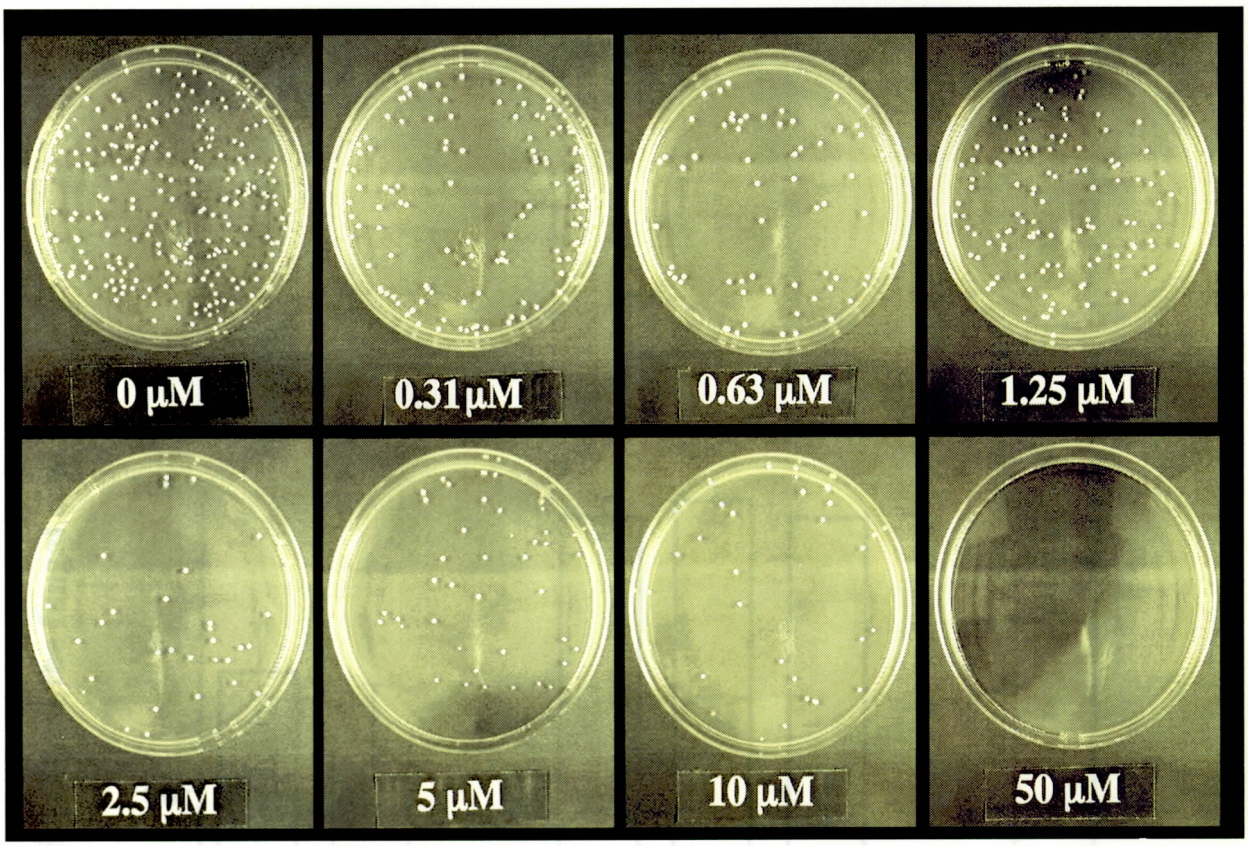
Fig. 2 Histatin-5 (50mM) induced loss of viability of *C. albicans* isolates. The data indicate mean loss of viability with standard deviations. *C. albicans* A1 and A2 are the oral isolates from HIV-positive patients, and A6, A8 and A10 are the oral isolates from HIV-negative patients. ATCC was used as a reference strain.

Table 1. Results of ANOVA on fungicidal effects

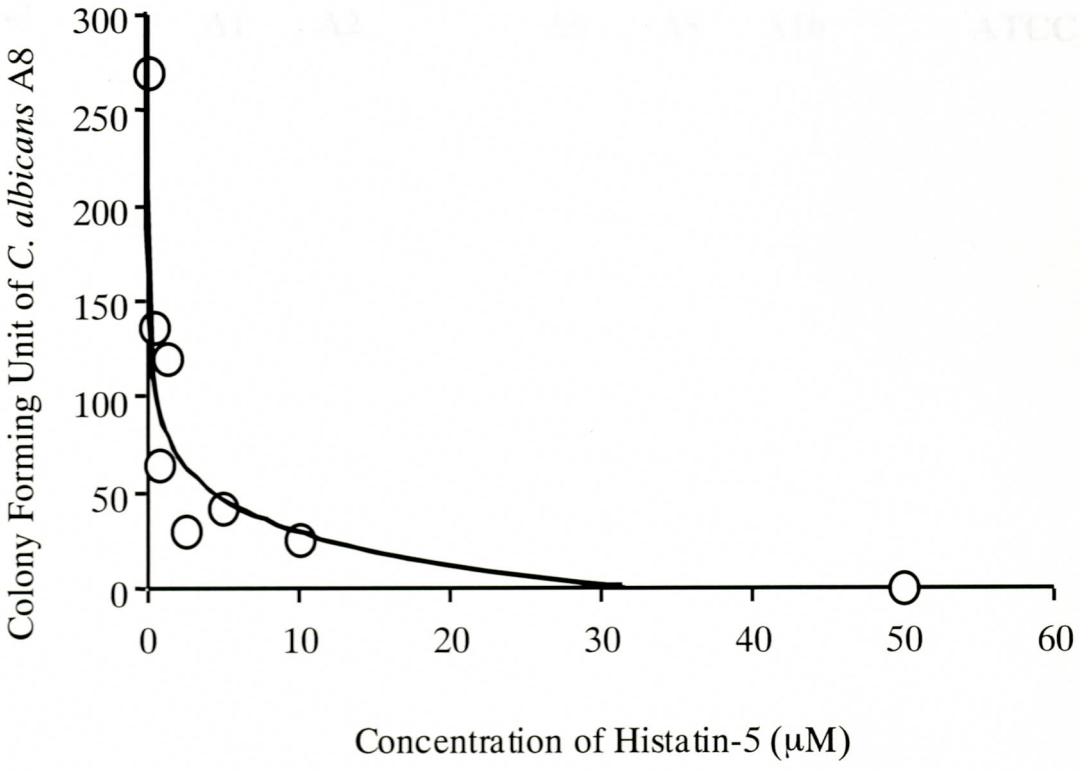
Fungicidal effects	S	f	V	F	p
A	**6930.50**	**5**	**1386.10**	**8.63**	**0.00000**
e	**2889.75**	**42**	**68.80**		
T	**9820.25**	**47**			

S, sum of squares; f, frequency; V, variance; F, F test; p, p-value

a)


0 μM
0.31μM
0.63 μM
1.25 μM
2.5 μM
5 μM
10 μM
50 μM

b)


Colony Forming Unit of C. albicans A8
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Concentration of Histatin-5 (μM)

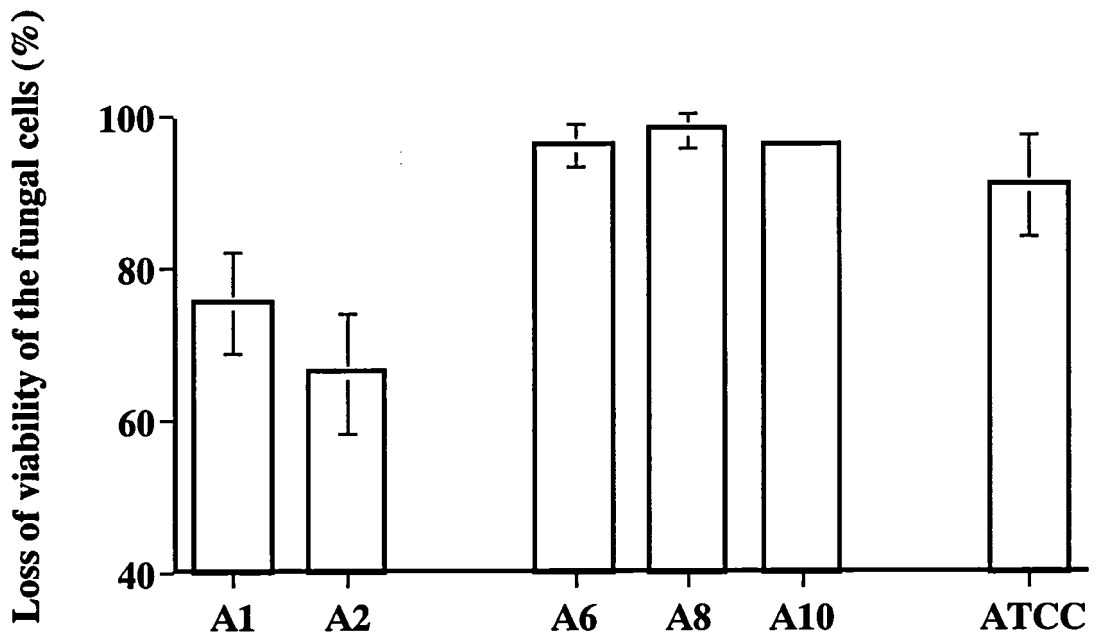


Fig. 2 Nikawa et al.