Genotype and phenotype of oral *Candida albicans* from patients infected with the human immunodeficiency virus

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***Candida albicans* has been shown to vary in its phenotypic expression with the progression of human immunodeficiency virus (HIV) infection. Isolates of *C. albicans* were obtained from 45 patients with HIV infection during the progression of their disease and differentiated using two methods. The first utilized the morphological characteristics of colonies, and the second method utilized a small portion of *C. albicans* DNA as a probe on Southern-transferred, *Eco*RI-digested *C. albicans* genomic DNA. In 67% of the patients a single strain of *C. albicans*, as determined by the DNA analysis, was isolated from each individual. The phenotypic expression of the genetically identical strains varied considerably over the experimental period with one morphotype being predominant. These results showed that the genotype of *C. albicans* persisted in the majority of HIV-infected individuals, but that the phenotypical expression of this strain changed. A novel finding in this study was that 18 strains of *C. albicans* had DNA which did not hybridize to the probe used.**

Keywords: *Candida albicans*, HIV infection, oral candidosis, genetic typing, phenotype

INTRODUCTION

The most common fungal disease seen in the oral cavity is candidosis and the *Candida* species most often involved is *C. albicans*, although *C. glabrata* and *C. tropicalis* are occasionally isolated (MacFarlane, 1990). Oral candidosis is a common feature of patients with human immunodeficiency virus (HIV) infection. In 17 studies the reported frequency of the occurrence of oral candidosis in HIV-infected patients ranged from 11% to 96%, with the majority of reports quoting frequencies greater than 50% (Holmstrup & Samaranayake, 1990). The frequency of isolation of *Candida* and clinical signs of oral candidosis have been shown to increase with advancing HIV infection (Torssander *et al.*, 1987; Kortling *et al.*, 1988). Candidosis of the oesophagus is one of the opportunistic infections seen in acquired immunodeficiency syndrome (AIDS) and its presence with HIV infection is diagnostic of AIDS according to the Centers of Disease Control (Atlanta, USA) surveillance definition (Greenspan *et al.*, 1990).

It has been postulated that differences in pathogenicity exist between strains of *C. albicans* and many methods have been developed to divide *C. albicans* into strains or subpopulations which reflect their pathogenic potential.

These strain-differentiating methods have also been used for epidemiological purposes (Merz, 1990). Several investigators have suggested that some strains of *C. albicans* have a greater propensity to cause systemic, nosocomial and superficial infections than do other strains (Kortling *et al.*, 1988; Brawner & Cutler, 1989).

The majority of the strain-differentiating methods are based on the phenotypic characteristics of the organism. One such method is based on the nature and extent of marginal fringing of streak colonies (Phongpaitchit *et al.*, 1987). When this method was used by Hunter *et al.* (1989) on isolates from fatal infections, 67% had a discontinuous fringe morphotype whereas this morphotype was seen in only 11% of strains isolated from other infections. These authors postulated that there may be an association between virulence and the morphotype of strains of *C. albicans*.

Serotyping of *C. albicans* divides the species into two groups, types A and B (Hasenclever & Mitchell, 1961a). Early studies using an animal model (Hasenclever & Mitchell, 1961b) did not show a correlation between serotype and virulence. More recent studies have shown that there is an increased frequency of 5-fluorocytosine resistance in serotype B (Auger *et al.*, 1979; Brawner *et al.*, 1992), and have demonstrated that there is an increased

pathogenic potential that correlates serotype B and immune status.

Recent advances in molecular biology have allowed the use of restriction enzyme analysis of genomic DNA to classify *C. albicans* at a subspecies level useful for epidemiological purposes (Magee *et al.*, 1987; Smith *et al.*, 1989; Matthews & Burnie, 1989; Monod *et al.*, 1990). The use of a species-specific repeated gene segment as a DNA probe in restriction enzyme analysis has enabled more highly detailed epidemiological studies because of the large number of different types defined by this technique (Fox *et al.*, 1989; Scherer & Stevens, 1988).

The aim of the current investigation was to assess the morphotype, serotype and genotype of oral mucosal candidal organisms in a group of HIV-infected patients over time to ascertain if these parameters changed during the experimental period.

METHODS

Patients. Sixty patients of the Fairfield Hospital for Infectious Diseases were entered into this study between November 1989 and April 1992 after approval was given by the Ethics Committee of that Hospital. All were diagnosed as group IV HIV infection according to the classification of the Centers for Disease Control (CDC), Atlanta, USA. After receiving informed consent, a complete oral examination was undertaken and oral samples were collected by the imprint culture method of Arendorf & Walker (1980) and the oral rinse technique outlined by Samaranayake *et al.* (1986) for laboratory analysis. The patients were reviewed during their normal medical appointments when further oral samples were taken. At each examination the most recent assay of the patients' CD4-positive T lymphocytes as a percentage of total lymphocytes (cells μl^{-1}) was recorded as an indication of immune status.

Identification of *Candida* species. Yeast colonies were speciated by three methods. Firstly, each isolate was assessed for the production of germ tubes after incubation in horse serum at 37 °C in a shaking water bath for 2 h. Secondly, they were assessed for the production of chlamydospores when inoculated under a sterile cover slip in the dark for 72 h on cornmeal agar (Oxoid) containing Tween 80 (Sigma). Finally, each isolate was tested for carbon assimilation by the API 20C AUX identification system (bioMerieux). Samples were separated as either *C. albicans* or *Candida* species other than *albicans*. The *C. albicans* samples were serotyped into group A or B by a slide agglutination test using the Iatron factor 6 rabbit antiserum (IF6) (#RM 302-4, Iatron Laboratories). All samples were inoculated into 2 ml of storage medium consisting of 100 ml Luria broth (10 g tryptone, 5 g yeast extract powder, 10 g NaCl in 1 l sterile deionized water) and 30.5 ml glycerol (BDH Chemicals) and stored at -20 °C until further analysis was performed. Only those patients who had *C. albicans* isolated on more than one occasion were used in this study.

Colony morphology. For morphotyping, stored yeast cells were resuscitated from -20 °C by culturing on Sabouraud's agar (Oxoid) at 37 °C for 48 h. Single colonies from these cultures were then streaked onto 2% (w/v) malt agar (Oxoid) and incubated at 30 °C. After 24 h the yeast cells were suspended in sterile deionized water to give a distinct turbidity corresponding to 10^7 - 10^8 cells ml^{-1} as described by Phongpaitchit *et al.* (1987). Agar plates containing 6% (w/v) malt extract and 2% (w/v) Difco special agar (Oxoid) were then inoculated with a single diametric streak of the test suspension using a sterile cotton wool swab. Cultures were incubated in the dark for 10 d at 30 °C after which the streak characteristics of each inoculum were determined. A modified three digit coding system of numbers was allocated to the features of the colony streaks as outlined by Hunter *et al.* (1989). The initial digit represents the distribution, the second the width and the third the texture of the fringe. The numbering system for the various attributes is outlined in Table 1.

Genetic typing. For genetic typing, stored yeast cells were initially cultured on Sabouraud's agar (Oxoid) at 37 °C for 24 h. A single yeast colony from these cultures was then inoculated into 10 ml YPD broth (1 g yeast extract, 2 g bactero-peptone and 2 g D-glucose in 100 ml sterile water) and incubated overnight in a shaking water bath at 37 °C. *Candida* DNA was prepared by the method of Scherer & Stevens (1987) except that the DNA was extracted five times with phenol (ICN Biomedicals)/chloroform (Prosana Laboratories) (1:1, v/v) prior to precipitation in 2 vols ethanol (Rhone-Poulenc Laboratories). Approximately 3 μg of DNA was digested with 5 units *Eco*RI (Boehringer Mannheim) overnight at 37 °C to ensure complete digestion. The DNA fragments were separated through a 0.7% (w/v) agarose gel in TBE buffer (89 mM Tris base, 89 mM Boric acid, 0.2 mM EDTA) for 18 h at 70 V. The DNA was transferred under vacuum to a positively charged nylon membrane (Boehringer Mannheim) which was then hybridized overnight in 50% (v/v) formamide, 0.9 M NaCl, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA, 0.3% SDS, and 100 μg denatured salmon sperm DNA ml^{-1} with a digoxigenin-labelled species-specific probe. The probe, 27A, is an *Eco*RI fragment of *C. albicans* cloned into the plasmid vector pUC18 and was kindly supplied by Dr S. Scherer, University of Minnesota, Minneapolis, USA. For labelling, the *Eco*RI fragment 27A was subcloned into plasmid pT7T3 (Pharmacia) and a digoxigenin-labelled RNA probe was prepared from the *Hind*III-digested plasmid with T7 RNA polymerase using a labelling kit supplied by Boehringer Mannheim. After hybridization the probe was detected using an alkaline phosphatase-

Table 1. System of coding of the morphological features

Feature	Code	Description
Fringe distribution	0	Absent
	1	Discontinuous; < 20% of margin
	2	Discontinuous; 20-50% of margin
	3	Discontinuous; > 50% of margin
	5	Continuous at the periphery or strands conspicuously fan shaped
Fringe width	7	Continuous; filamentous outgrowths parallel
	0	Absent
	2	< 2 mm
	3	2-5 mm
	5	> 5 mm
Fringe texture	0	Absent
	1	Very coarse
	2	Coarse
	3	Intermediate
	4	Fine

Fig. 1. Southern blot hybridization of *EcoRI*-digested *Candida* DNA from randomly selected oral isolates of 15 patients.

Fig. 2. Southern blot hybridization of *EcoRI*-digested *Candida* DNA from consecutive oral isolates. The lanes are grouped above for the same patient and the numbers below are the times in months at which the isolates were taken. The isolates taken at 0 and 12 months from patient 18 and the isolate taken at 14 months from patient 22 were genetically distinct from other isolates of those patients. These variations were reproducible when different colonies taken at the same time were processed.

conjugated antibody (Boehringer Mannheim) according to the manufacturer's instructions. This resulted in the ability to use a non-radioactive technique with markedly reduced background staining.

RESULTS

The 60 patients in this study have all died. The average survival time since diagnosis of AIDS (group IV HIV infection, CDC) (Greenspan *et al.*, 1990) was 20.5 months with a range of 3–36 months.

Forty-five of the 60 patients were included for further analysis on the basis that *C. albicans* was isolated on more than one occasion during the experimental period. Of the remaining 15 patients, four had *C. glabrata*, two had *C.*

krusei and two had *C. tropicalis* isolated, four had *C. albicans* isolated only once and three patients did not have any yeast isolated from their oral cavities during the experimental period. The 45 patients analysed had a total of 295 samples (mean = 6.5 samples per patient) taken at various intervals ranging from 2 to 24 months (mean = 9.3 months). Of these 45 patients, 23 (51%) had at some stage during the experimental period signs and symptoms of oral candidosis as diagnosed by the criteria of Greenspan *et al.* (1990). These patients were treated with either amphotericin, nystatin or fluconazole. Twenty of these 23 patients were in a concurrent fluconazole trial which consisted of 100 mg d⁻¹ for 1 week and a maintenance dose of 50 mg daily for approximately 2 months. Recurrence of oral candidosis occurred in 48% (11/23) of

Fig. 3. Southern blot hybridization of *EcoRI*-digested *Candida* DNA from consecutive oral isolates. The lanes are grouped above for the same patient and the numbers below are the times in months at which the isolates were taken. The isolates from patient 9 have two bands at 3 kb whilst those of patient 10 have one. Two of the isolates from patient 10, 0 and 7 months, did not show any hybridization with the DNA probe used.

Fig. 4. Four morphotypes taken at different times from the same patient over a period of 9 months. The four morphotypes are (a) 123, (b) 731, (c) 522 and (d) 534.

these patients after the medication was ceased. The average time for recurrence was 5.2 months (range 2–9.5 months).

Genetic analysis

Genetic typing of the *C. albicans* isolates by Southern blot hybridization showed significant variation in the banding patterns between isolates from different patients. This is illustrated in Fig. 1 where the DNA profiles of randomly selected *C. albicans* isolates from 15 patients are shown. This suggested the existence of a large number of different genetic types within the species which indicated this technique is useful for determining the identity of isolates. Analyses of all *C. albicans* isolates by genetic typing revealed that there were 60 genetically different strains isolated from the 45 patients. The majority (30/45) of the patients harboured one strain unique to that patient and this strain persisted over the experimental period (Fig. 2). Fifteen of the 45 patients (33%) had two genetically different strains isolated during the experimental period. Of the 149 isolates from these 15 patients, 16% (25/149) were genetically different from the predominant strain. In each patient where another strain was isolated, the isolation was not consistently related to episodes of either clinical oral candidosis or antifungal therapy. No patient had more than two genetically different strains isolated during the experimental period. In 20% (9/45) of patients, 18 of their 74 isolates (24%) had genomes which did not show any hybridization with the DNA probe used (Fig. 3). Each of these strains was regrown on three occasions and the species identification repeated and checked by an independent observer. On each of these three occasions the DNA was extracted before repeating genetic typing as outlined above. All of the 18 strains were shown to be *C. albicans* whose DNA did not hybridize to the probe (27A) used in this study.

The slide agglutination test for serotyping *C. albicans* revealed that 88% (53/60) of the genetically different strains isolated were serotype A while the remaining 12% (7/60) were serotype B.

Morphological analysis

Each patient examined had two or more *C. albicans* morphotypes isolated at different times during the study period. The average number of different morphotypes isolated from each patient was 4.6 with a range of between 2 and 11. As an example, the morphotypes shown in Fig. 4 were isolated from the same patient over a 9 month period. From the 45 patients, 37 different morphotypes were isolated. Thirty-seven of the 45 patients (82%) had the morphotype 754 isolated at least once during the period of examination. This morphotype occurred in 27.5% of all isolates, whereas all other morphotypes occurred at a frequency less than 7%.

The 295 isolates were divided into groups according to the percentage of CD4-positive T lymphocytes against total lymphocytes (%T4) at the time when each sample

was taken. Eighty-three of the 295 isolates (28%) were taken when the %T4 was less than 5%, while 47% (139/295) were taken when the %T4 was less than 10%. χ^2 analysis of the morphotypes of these groups (%T4 < 5%, v. %T4 > 5%; and %T4 < 10%, v. %T4 > 10%) showed no significance ($P = 0.1$ and $P = 0.25$, respectively) in the occurrence of any one morphotype.

Similarly, when all the discontinuous fringe morphotypes were combined, and the distribution of this combination was compared according to the %T4 of each patient as grouped above, no significant relationship ($P = 0.35$ and $P = 0.5$, respectively) between the discontinuous fringe morphotype and the declining immune status of the patients was found.

DISCUSSION

The rate of multiple isolation of *C. albicans* from the oral cavity of HIV-infected patients in the present study was 67% which is similar to that of previous studies (Korting *et al.*, 1988; Torssander *et al.*, 1987). The finding in the present study that 51% (23/45) of patients developed clinical signs of oral candidosis also correlated well with the study of Korting *et al.* (1988) who found that 54.2% (13/24) of their patients with AIDS were observed to develop clinical oral candidosis. A higher prevalence of 70% (21/30) of oral candidosis was reported by Miyasaki *et al.* (1992) in their study of HIV seropositive subjects. Such discrepancy may be due to patient selection or degree of immunosuppression of the selected group. In the present study the patients were chosen randomly from individuals regularly attending an out-patient clinic for HIV infection and had been diagnosed as category IV HIV infection (CDC) at the outset of the investigation. In the study of Miyasaki *et al.* (1992), the study group was not separated into any HIV staging classification and thus the degree of immunosuppression of this group was difficult to assess.

Several studies have investigated the genetic relatedness of *C. albicans* isolates by the use of probes 27A (Fox *et al.*, 1989) and Ca3 (Soll *et al.*, 1991; Miyasaki *et al.*, 1992) which represent the same family of repeated sequences (Sadhu *et al.*, 1991). The observation of the genetic relatedness of the *C. albicans* strains from HIV-infected patients reported here differs from those previous studies.

We found that two-thirds of the patients had only one patient-specific strain isolated throughout the course of this study and that one-third had two genetically different strains isolated. This contrasts with the findings of Miyasaki *et al.* (1992) who found that none of their 30 patients had more than one strain of *C. albicans* isolated over the experimental period. Powderly *et al.* (1992) also found that by using the same genetic test as described here, all 39 isolates from their HIV-infected patients were a unique genetic type. This research, however, did not assess the temporal presence of *C. albicans* strains in these patients. Furthermore, Fox *et al.* (1989) showed that the DNA profile pattern using the 27A probe was identical in

the *C. albicans* isolated serially (2–18 months) from eight hospitalized, immunocompromised patients. In contrast, in a recent study using multilocus enzyme electrophoresis patterns of *C. albicans* isolates from bone marrow transplant patients over a 13 month period, Caugant & Sandven (1993) found that three of their seven patients were colonized by more than one strain. This finding is similar to the results of our study as the number of times a genetically different strain was isolated from the group of patients who harboured two strains in the present study was small (16%). The difference between these results may reflect the number of colonies sampled at each time point and the number of oral samples taken from each patient. The second strain may only be present in small numbers in the oral cavity by comparison with the more predominant strain. Throughout this study only one randomly chosen colony was analysed at any one sampling.

The fact that the second, less predominant strain was not consistently isolated during clinical oral lesions, or during treatment with antifungal medication suggested that this strain is not more virulent or more drug resistant. Similar observations have been made in other studies (Miyasaki *et al.*, 1992; Whelan *et al.*, 1990). Recently, Gallagher *et al.* (1992) have shown that phenotypically switched variants of *C. albicans* can develop decreased azole susceptibility even though these strains remained genetically identical. These authors have shown, thereby, that it is not necessary to have alterations in the genetic type of strain for there to be changes in the drug susceptibility of the organism. Blumberg *et al.* (1992) provided clinical evidence to support this contention by showing that the same genetic strain of *C. albicans* was isolated from a patient with fungemia in whom fluconazole failed to eradicate the organism. There was a marked decrease in the *in vitro* susceptibility of these genetically identical strains throughout the course of this patient's infection.

The lack of hybridization of 18 strains of *C. albicans* with the species-specific 27A probe has not previously been reported. All of these 18 strains were regrown, speciated independently by the use of the API 20C AUX identification system (bioMérieux). The DNA was exhaustively purified during the extraction procedure before Southern blot hybridization. In each case the strains were *C. albicans* and their DNA failed to hybridize with the 27A probe. This procedure was repeated on three occasions when the original findings were confirmed. The most likely explanations are that the isolates may be another species of *Candida* which are phenotypically identical to *C. albicans* but genetically distinct, or that the probe target is not present in all strains of *C. albicans*.

The results of the serotyping of the *C. albicans* isolates presented here (88% serotype A and 12% serotype B) were similar to the occurrence of serotypes from early studies (Martin & Lamb, 1982) but differed from recent studies of the prevalence of serotype B in the immunocompromised host (Brawner *et al.*, 1992; Brawner & Cutler, 1989). In a study comparing different methods of serotyping *C. albicans*, Brawner (1991) found that the IF6

rabbit antiserum may slant results in favour of type A prevalence by as much as 24% due to autoagglutination of serotype B isolates. This may have increased the prevalence of serotype A in the present study.

The wide variation in the morphotypes of each patient's genetically identical *C. albicans* isolates (mean = 4.6 per patient) presented here raises the question of the reproducibility of this technique. Hunter & Fraser (1989) recently analysed the discrimination and reproducibility of four *C. albicans* typing methods including morphotyping. They found that the reproducibility of the colonial fringe characteristic in their *in vitro* study was 89%. The results of our study suggest a much lower reproducibility which may reflect the phenomena of phenotypic switching (Anderson & Soll, 1987; Slutsky *et al.*, 1987) or subtle phenotypic changes in the organism as a result of local environmental pressure.

The high prevalence of the morphotype 754 in this study (82% of patients and 27.5% of total isolates) does not have a ready explanation. A recent study of morphotypes of *C. albicans* from HIV-infected patients by Oliver & Reade (1993) found the morphotype 724 to be isolated from 52% of ARC (AIDS-related complex) and AIDS patients, whilst this morphotype was not isolated from HIV asymptomatic patients. These two morphotypes (754 and 724) differ only in the width of the fringe which may reflect technical variations, but both had continuous fine fringes. Oliver & Reade (1992) postulated that there may be an association between progressive immunosuppression from HIV posterity to AIDS and a lack of antibody response to this morphotype and that this change in phenotypic expression may have predictive significance for subsequent disease progression. The findings from the present study showed that morphotype 754 was common in category IV HIV-infected patients, but was not related to their degree of immunosuppression as expressed by the percentage CD4-positive T lymphocytes.

Hunter *et al.* (1989) suggested that there was an association between the discontinuous fringe morphotype and fatal deep infections. They made this association by combining the morphotypes with the initial digit 1, 2, 3 and 5. When the same combination of morphotypes was made in our study there was no significant relationship with declining immune status. This result supports the theory of Hunter *et al.* (1989) that the fringe characteristic is a reflection of anatomical source of the isolate rather than reflecting an association with host immune status. Whether these morphotypes are directly related to any increased virulence of the organism remains unclear and warrants further research. It may be that the changing morphotypes of genetically identical strains of *C. albicans* within individuals over time represents switched variants, and in the light of the research by Gallagher *et al.* (1992) and Blumberg *et al.* (1992) this may have a bearing on changes in the susceptibility of these strains to antifungal agents.

The results of the present study showed that the same genetic strain of oral *C. albicans* isolated persisted in the majority of category IV HIV-infected individuals, but

that the phenotypical expression of this strain changed with time.

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