MYCOLOGY

Heterogeneity of oral isolates of *Candida albicans* in HIV-positive patients: correlation between candidal carriage, karyotype and disease stage

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Opportunist infections involving *Candida albicans* often develop in HIV-positive patients and oral lesions tend to become more frequent as the disease progresses. Previous studies have shown contrasting results concerning the variability of the pulsed-field gel electrophoresis (PFGE) subtypes of *C. albicans* observed in HIV-positive patients. Carriage of *C. albicans* was determined by an oral rinse technique; 41 strains of *C. albicans* (78% serotype A and 22% serotype B) were isolated. There was a direct correlation between candidal load (cfu/ml) and the blood HIV load, whereas there was an inverse correlation with the stage of disease and the CD4 cell counts. The PFGE patterns of isolates were variable with regard to the number and positions of bands. The variability of the band sizes in some run positions showed a Gaussian distribution. Generally, the most frequent size variants were associated with the strains with the highest cfu/ml and lowest CD4 counts (≤ 200 cells/ μ l). These findings suggest a possible strain selection over time during disease progression, especially in HIV-positive subjects with low CD4 counts.

Introduction

Oral candidosis is the most frequent opportunist infection in HIV-infected patients [1–4]. Clinically, there are three forms of oral candidosis: pseudomembranous, erythematous and angular cheilitis [1–5]. *Candida albicans* is usually isolated, whereas non-*albicans* species, such as *C. glabrata*, *C. parapsilosis* and *C. tropicalis* are encountered less often [4, 5]. Previous studies have reported differences in *C. albicans* serotype distribution in certain populations, including HIV-positive subjects. The two principle serotypes of *C. albicans* are serotypes A and B, although the individual pathogenic role of each remains uncertain [6–10]. Phenotypic methods have revealed strain differences in *C. albicans* [11–13], but there is still no clear relevance to strain virulence. More recently, genotyping of *C. albicans* has been performed in an attempt to define pathogenic associations. These techniques include pulsed-field gel electrophoresis

(PFGE) for karyotyping [14, 15]. In fact, PFGE has been employed mainly to highlight inter- and intra-species differences. A PFGE variant, contour-clamped gel electrophoresis (CHEF) is considered the best approach for strain differentiation [16, 17]. However, extensive PFGE studies on various micro-organisms have not been completely satisfactory because of lack of standardisation of methods and this has produced conflicting results in different laboratories [14–17].

Genetic differences between strains of *C. albicans* isolated from HIV-positive patients and strains from HIV-negative subjects have been reported. In particular, karyotype variability was found to be significantly reduced in the HIV-positive group [18, 19]. In contrast, other workers have found a wide spectrum of *C. albicans* karyotypes isolated from HIV-positive patients [20]. Furthermore, there do not appear to have been any reports describing the possible association between the band size variants of the karyotype patterns, the virulence of the *C. albicans* strains and the stage of the patient's HIV infection, CD4 levels and HIV viral load.

The aim of the present study was to explore the possibility of a relationship between the level of *C.*

Received 14 Oct. 1999; revised version received 29 March 2000; accepted 29 March 2000.

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albicans carriage and the different karyotype patterns. It also aimed to relate *C. albicans* carriage, serotype and karyotype to disease stage, CD4 cell levels and blood HIV load.

Materials and methods

Patients

Untreated patients with *C. albicans* colonisation were enrolled into the study at the Centre for Sexually Transmitted Diseases of the San Gallicano Institute of Rome. Staging of disease was according to the criteria established by the Centers for Disease Control in 1993 [21].

Culture and identification

Quantitative candidal carriage was determined by asking the patient to rinse their mouth with 20 ml of saline for 30 s, as described previously [22]. After vigorous shaking for an additional 30 s, the rinse was serially diluted (1 in 2 with sterile saline) and 100 µl of each dilution were inoculated on to plates of CHROMagar [22] and incubated at 37°C for 48 h. Colonies provisionally identified as *C. albicans* were subcultured twice on to non-selective plates of Sabouraud's Agar (Biotec) for purity. Identification of *C. albicans* was confirmed by germ-tube formation in fetal calf serum and a commercial identification kit (API ID 32C; bioMérieux, Charbonniere Les Bains, France).

Isolates of *C. albicans* were subcultured in Sabouraud's Broth (Oxoid) and incubated overnight at 37°C in a shaking incubator. A control strain, *C. albicans* ATCC 24433, was also cultured under the same conditions.

DNA was extracted from *C. albicans* strains with a commercially available kit (Enzyme module group IV; BioRad Laboratories, Tucson, USA). Briefly, DNA was extracted with 600 µl of Sabouraud's broth suspension with lyticase, as described previously [23–25]. Optimal extraction was obtained with prolonged lysis time of the *C. albicans* plugs, extending the incubation with lyticase overnight. After overnight proteinase K deproteinisation (BioRad Laboratories), the plugs were put into wells of agarose 1% gel in 0.5× Tris Borate EDTA (Sigma). In each electrophoresis gel, plugs containing *Saccharomyces cerevisiae* as ladder standard preparation (size range 225–2200 kb), *C. albicans* ATCC 24433 DNA (BioRad Laboratories) and *Hansenula wingei* chromosomal DNA (1.05–2.35 Mb) were added as controls. The running time was fixed at 48 h, selecting the appropriate program of the GenePath power module (BioRad Laboratories). After staining with ethidium bromide, the gel was photographed with BioRad's Polaroid Documentation System (BioRad Laboratories). Finally, the DNA fragment sizes were calculated by plotting the molecular sizes and the

migration distances of *S. cerevisiae* DNA ladders (BioRad Laboratories) included in each gel [9, 26].

Serotyping of *C. albicans* was performed with the Candida Check kit (Alfa Biotech, Milan, Italy) with five antisera specific for the candida cell-wall antigens 4, 5, 6, 13 and 13b [6, 8, 10]. Biochemical patterns were defined by the biochemical tests included in the API ID 32C kit.

CD4 cell counts

The number of CD4-positive cells in the blood samples was measured by flow cytometry (Cytoron Absolute, Ortho, Ortho Diagnostic Systems) and specific monoclonal antibodies (Becton Dickinson, USA).

Determination of HIV load

Viral load quantification (quantitative PCR) was performed by the Amplicor HIV-1 Monitor test (Roche Diagnostic System; Branchburg, NJ, USA), according to the manufacturer's instructions.

Statistical analysis

The χ^2 test and Fisher's exact test were employed, as appropriate, to analyse the differences in the results. Contingency tables for groups of less than two were also employed. Quantitative comparisons were obtained by means of non-parametric tests – Mann-Whitney or Friedman variance analysis. Correlations were calculated by employing the Spearman's rank correlation test.

Results

A total of 41 HIV-positive patients, 36 males and 5 females with a median age of 34.5 years (range 24–57), was enrolled into the study at the AIDS Centre of the San Gallicano Institute of Rome. Four patients were at disease stage A1, two at A2, eight at B1, 17 at B2, one at C2 and nine at C3. None of the subjects was an intravenous drug user. The median CD4 count was 455 cells/µl (range 312–1168 cells/µl) and median blood viral load was 0.9 copies/ml (range 0.3–2.1 copies/ml). Twelve patients were newly diagnosed and had not received antifungal drugs, nine further newly diagnosed patients had not received antiviral drugs. Eighteen subjects interrupted their antifungal therapy because of toxicity, 11 interrupted their antiviral therapy because of severe side effects, 11 refused antifungal therapy and 21 refused antiviral therapy. Twenty-five male patients were homo-bisexual and 11 were multiple-partner heterosexuals. The five female patients were sexual partners of intravenous drug users. Informed consent was obtained from all patients.

Only subjects with *C. albicans* were enrolled into the

study. The characteristics of the *C. albicans* strains are shown in Table 1. The biochemical identification indices obtained for the different *C. albicans* strains were always >85%.

Although, overall, serotype A predominated (78% of isolates), serotype B was relatively more frequent in advanced stages of disease (0% in stage A, 35% in stages B and C). However, this difference was not statistically significant.

The counts of *C. albicans* in the 41 patients differed widely (Table 1). Colony numbers and the blood viral loads were evaluated after log₁₀ transformation. The level of *C. albicans* carriage was significantly greater in patients with CD4 counts ≤200 cells/μl than in those with higher CD4 counts ($p < 0.001$). No significant differences were observed by subdividing the patients with CD4 counts >200 cells/μl into groups with >200–≤450 cells/μl and >450 cells/μl. The median log₁₀ cfu/ml of *C. albicans* increased significantly ($p < 0.001$) with the stage of disease: stage A1/A2, 1.65 (range 1.18–2.41); stage B1/B2, 2.0 (range 1.30–1.70) and stage C2/C3, 3.40 (range 2.41–3.70).

A strong correlation was observed between the log₁₀ cfu/ml of *C. albicans* and the log₁₀ viral load copies/ml (Fig. 1; $r = 0.85$, $p < 0.001$). A significant inverse correlation was observed between the log₁₀ viral load and the CD4 counts ($r = -0.53$, $p < 0.001$), and the log₁₀ cfu/ml was inversely correlated with CD4 counts ($r = -0.67$, $p < 0.001$).

The PFGE (CHEF) patterns of the 41 strains are shown schematically in Fig. 2. The DNA sizes of the different bands were calculated by measuring the distance of each band from the start of the run. The sum of the partial sizes permitted calculation of the overall sizes. No relationships between the number of the bands or the overall DNA sizes and other variables were observed (data not shown).

No identical PFGE patterns were obtained with the 41 strains of *C. albicans* studied, indicating a high level of

karyotype variability. The restricted number of patterns previously reported [20] was not observed (Fig. 2). The first band was the widest and most constant in size; the remaining bands showed frequent size variations. When analysing the variability of the second, third and fourth band, a Gaussian-like distribution of these sizes was observed (Table 2). The most frequent size variants for each of the three bands considered were significantly associated with the strains showing high levels of cfu/ml. The relationship between the median log₁₀ cfu/ml and the different positions for each band is shown in Table 2. Strains with the less frequent band sizes showed lower levels of cfu/ml and these differences were statistically significant when the bands were grouped.

The data shown in Table 3 suggest that the less frequent size variants of bands II, III, and IV were associated with strains isolated from subjects with CD4 counts >200 cells/μl.

Discussion

In the present study, *C. albicans* serotype B was encountered in stages B and C of the CDC classification rather than stage A, as previously reported for HIV-positive patients [9, 10, 27, 28]. Furthermore, an evident correlation was found between the quantitative data regarding the oral colonisation by *C. albicans* in HIV-positive patients and their viral load. Such a finding does not appear to have been reported previously [1–5, 29]. The lack of correlation with other studies may be due in part to the less stringent conditions employed elsewhere. The absence of anti-fungal and antiviral treatment [11], the number of patients observed and the strict standardisation of the counting procedure in the present study may have influenced the findings. The correlation between the HIV log₁₀ viral load and *C. albicans* log₁₀ cfu results in two further significant associations, i.e., the HIV log₁₀ viral load *versus* the CD4 numbers (inverse correlation) and the log₁₀ cfu/ml *versus* the CD4 numbers (inverse correlation). As expected, the stages

Table 1. Characteristics of 41 strains of *C. albicans* isolated from the mouths of 41 HIV-positive patients.

A:B serotype ratio	32:9
Median (range) log ₁₀ cfu/ml	
All patients	2.10 (1.18–3.70)
Patients with CD4 count ≤200	3.40 (2.41–3.70)
Patients with CD4 count 200–450*	1.95 (1.40–3.17)
Patients with CD4 count >450*	2.00 (1.18–3.35)
Median (range) number of PFGE bands	7.5 (6–9)
Number of strains (%) with	
6 bands	1 (2.4%)
7 bands	25 (61.0%)
8 bands	14 (34.4%)
9 bands	1 (2.4%)
Median (range) value of total DNA size:	12 500 (10 300–15 750) kb

Fig. 1. Correlation between $\log_{10}$ HIV viral load and $\log_{10}$ cfu/ml of *C. albicans*.

inversely correlated with the CD4 counts and, therefore, the HIV $\log_{10}$ viral load and the *C. albicans* $\log_{10}$ cfu/ml also directly correlated with the stage shift.

The 41 strains of *C. albicans* showed highly homogeneous patterns in biochemical analysis. An initial attempt to subclassify these strains, using their sensitivity tests, revealed 17 different subgroups (data not shown) with no apparent associations with the other variables considered. To enhance the possibility of analysing the strains' characteristics, the karyotypic variability was evaluated. The results obtained by PFGE (CHEF) permitted calculation of the number and definition of the band patterns, as well as the overall DNA size for each strain. These three variables did not show any correlation with the *C. albicans* serotype, the viral load, the number of cfu/ml or the stage of disease in the present study. Differences between oral strains of HIV-positive and HIV-negative subjects, mainly represented by more restricted band patterns in HIV-positive individuals, has been reported [18], although Lupetti *et al.* reported contrary results [20]. Lupetti *et al.* defined seven karyotypes based on the individual strain band positions on the gel. When this classification was applied to the samples in the

present study, some of the band patterns described were recognised, but in a context of high variability, which was more complex than that shown by Lupetti *et al.* Therefore, this kind of classification was not employed in the present study.

One of the aims of the present study was to determine if differences reported between HIV-positive and HIV-negative subjects could also be observed between HIV-positive individuals with conserved or altered immunity. For this purpose, three groups of subjects were considered: those with blood CD4 counts of ≤ 200 cells/ μ l, those ranging between >200 and ≤ 450 cells/ μ l and those with >450 cells/ μ l. In terms of band pattern, no difference was observed between these three groups (data not shown).

The position of individual bands was analysed. The distributions of the band size variants of the second, third and fourth band followed a Gaussian-type behaviour. Interestingly, the minus-variant and plus-variant (less frequent) sizes generally were associated with the lowest cfu/ml of the *C. albicans* strains. As the lowest counts of *C. albicans* were observed in individuals with conserved CD4 cell counts, the study

Fig. 2. PFGE patterns of 41 strains of *C. albicans* isolated from the mouths of 41 HIV-positive patients.

Table 2. Relationship between the median $\log_{10}$ cfu/ml and variation of band size (analysis restricted to the second, third and fourth band)

Band	Number of strains	Size of bands (kb)	Median (range) $\log_{10}$ cfu/ml	Median (range) $\log_{10}$ cfu/ml with grouping of bands
Second	1	NB	1.70 (1.7–1.7)	1.74 (1.4–2.08)*
	2	2350	1.80 (1.6–2.0)	
	3	2400	1.79 (1.4–2.1)	
	30	2450	2.76 (1.3–3.7)	2.76 (1.3–3.7)*
	5	2500	2.0 (1.18–2.89)	2.0 (1.18–2.89)*
Third	1	1700	1.70 (1.7–1.7)	1.8 (1.18–3.4)†
	4	1750	1.70 (1.6–1.9)	
	9	1800	2.08 (1.18–3.4)	
	17	1850	3.0 (1.4–3.7)	3.0 (1.4–3.7)†
	6	1900	3.37 (1.78–3.4)	
	2	1950	2.30 (2.19–2.41)	1.92 (1.3–2.41)†
	2	2000	1.48 (1.30–1.65)	
Fourth	4	NB	1.74 (1.6–3.0)	1.70 (1.4–3.0)‡
	4	1550	1.70 (1.4–1.88)	
	10	1600	2.94 (1.18–3.70)	3.0 (1.18–3.7)‡
	9	1650	3.0 (1.7–3.7)	
	9	1700	2.08 (1.3–3.4)	2.14 (1.3–3.4)‡
	5	1750	2.19 (1.6–2.41)	

NB, no band.

* $p = 0.025$; † $p = 0.01$, ‡ $p = 0.01$.

Table 3. Distribution of band sizes in relation to CD4 count

		Number of strains with DNA band size (kb)						
CD4 count/ μ l		NB	2350	2400	2450*	2500	Total	
Second band	≤ 200	0	0	0	10	0	10	
	$>200-\leq 450$	0	1	3	10	0	14	
	>450	1	1	0	10	5	17	
	Total	1	2	3	30	5	41	
		1700	1750	1800*	1850*	1900*	1950	2000
Third band	≤ 200	0	0	2	6	2	0	0
	$>200-\leq 450$	1	2	3	5	2	1	0
	>450	0	2	4	6	2	1	2
	Total	1	4	9	17	6	2	2
		NB	1550	1600*	1650*	1700*	1750*	Total
Fourth band	≤ 200	0	0	3	3	3	1	10
	$>200-\leq 450$	1	2	2	6	2	1	14
	>450	3	2	5	0	4	3	17
	Total	4	4	10	9	9	5	41

NB, no band.

*Band sizes common to patients with ≤ 200 CD4 cells/ μ l or >200 CD4 cells/ μ l. Difference between marked and no marked groups, $p = 0.0015$.

analysed whether these non-frequent bands were associated with high CD4 levels. The strains obtained from subjects with CD4 cells ≤ 200 μ l had a more restricted band size variability for all the three bands than strains from patients with a CD4 count of >200 cells/ μ l. There were no differences between subjects with CD4 counts of $>200-\leq 450$ cells/ μ l and those with counts of >450 / μ l.

Previous studies stressed that HIV-positive subjects generally presented with the same *C. albicans* strains until the CD4 count was >200 cells/ μ l, while strains showed differences below this CD4 level [4, 18] suggesting a selection of strains over time. The findings presented here support the possibility that strain selection occurs during disease progression in HIV-positive patients and that the surviving strains are those characterised by the most frequent band size variant patterns.

A possible relationship between antifungal therapy and karyotype patterns or band size variants also merits consideration. The study population contained subjects who were not taking antifungal therapy at the time of observation, even if some had been treated previously. No relationship was observed between previous antifungal treatment and the karyotype patterns. Band size variants (although not statistically significant) similar to those found in patients with CD4 counts ≤ 200 cells/ μ l were also observed in the group which had previously received antifungal therapy (data not shown).

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