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Susceptibility of *Candida dubliniensis* to Salivary Histatin 3

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Candida dubliniensis **is a recently described *Candida* species associated with oral candidiasis in human immunodeficiency virus (HIV)-infected patients and patients with AIDS. The majority of *C. dubliniensis* clinical isolates tested to date are susceptible to the commonly used antifungal drugs, including fluconazole, ketoconazole, itraconazole, and amphotericin B. However, the appearance of fluconazole-resistant *C. dubliniensis* strains in this patient group is increasing. Histatins are a family of basic histidine-rich proteins present in human saliva which have therapeutic potential in the treatment of oral candidiasis. The mechanism of action of histatin is distinct from that of commonly used azole and polyene drugs. Characterization of the antifungal activity of histatin has mainly been carried out using *C. albicans* but it is also effective in killing *C. glabrata* and *C. krusei*. Here we report that *C. dubliniensis* is also susceptible to killing by histatin 3. The concentration of histatin 3 giving 50% killing (the IC_{50} value) ranged from 0.043 to 0.196 mg/ml among different strains of *C. dubliniensis*. The least-susceptible *C. dubliniensis* strain, P9224, was found to internalize histatin at a lower rate than the *C. albicans* reference strain CA132A. The dissociation constant (K_d) for the least-susceptible strain (*C. dubliniensis* 9224) was ninefold higher than that for the *C. albicans* reference strain. These results suggest that histatin 3 may have potential as an effective antifungal agent, particularly in the treatment of oral candidiasis in HIV-infected patients and patients with AIDS in which resistance to the commonly used antifungal drug fluconazole has emerged.**

Histatins are a family of low-molecular-weight, cationic, histidine-rich peptides that are found in human saliva and which have potent and broad-spectrum antifungal activity. A number of histatin peptides have now been identified in saliva. Histatin 1 and histatin 3 are the gene products of the *HIS1* and *HIS2* genes, respectively, while histatin 2 and histatins 4 to 12 are their proteolytic cleavage products. One of the first in vivo observations on the role of histatin was that the concentration of histatins was greatly reduced in a group of human immunodeficiency virus (HIV)-infected patients with oral candidiasis (16). Clinical observations in other groups suggest that patients with inherently low levels of salivary histatins are predisposed to oral carriage of yeasts; however, the expression of histatins may be upregulated in response to actual candidal infection (2, 13). Histatin 5 is the most potent of the histatins in killing the blastospore and germinated forms of *Candida albicans*, followed by histatin 3, although histatin 3 is the most efficient in inhibiting germination of blastoconidia (37). Characterization of the antifungal activity of the histatins has been performed mainly with *Candida albicans*, the most common and the most pathogenic oral *Candida* species (5). However, histatins are also active against other yeasts and fungi—including *Candida glabrata*, *Candida krusei*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* (11, 28, 29, 35, 37)—and some bacterial species—including *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans* (18, 22). The mechanism of action of histatin, though not yet fully elucidated, involves pathways distinct from those involved in the mechanisms of action of commonly used antifungal drugs, such as polyenes and azole antifungals. Importantly, histatin 5 has been found to be active against amphotericin-resistant and azole-resistant *Candida* isolates and species (12). The current proposal on the sequence of events leading to histatin-mediated cell death is as follows: (i) binding of histatin to a fungal membrane receptor, (ii) translocation of histatin across the membrane, (iii) release of histatin into the intracellular compartment, (iv) interaction of histatin with cellular targets, and (iv) release of ATP (7, 11, 15, 39)

Candida dubliniensis is a recently described *Candida* species associated with oral colonization and infection in HIV-infected patients and patients with AIDS (36). More recently, it has been associated with oral carriage and infection in HIV-negative individuals and has also been recovered from a variety of specimens from nonoral sites, including the vagina, the respiratory tract, urine, sputum, feces, and blood (3, 4, 19, 20, 24, 27, 32, 36; D. Marriott, M. Laxton, and J. Harkness, Letter, Emerg. Infect. Dis. **7:**479, 2001). *C. dubliniensis* is phylogenetically closely related to *C. albicans* and has a worldwide distribution (6). The majority of *C. dubliniensis* isolates studied to date are susceptible to commonly used azole and polyene antifungal drugs, including ketoconazole, fluconazole, itraconazole and amphotericin B (14, 17, 20, 21, 25). However, resistance to fluconazole has been reported in clinical isolates (20), and studies have shown that a stable fluconazole-resistance phenotype associated with up-regulation of multidrug transporters can be generated following sequential exposure of *C. dubliniensis* isolates to increasing fluconazole concentrations in vitro (20). Azole antifungal drug resistance has been reported widely in *C. albicans* and other *Candida* species (8, 24, 38). The emergence of resistant fungal strains and the availability of

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TABLE 1. Histatin IC_{50}s determined for a range of *C. dubliniensis* strains from different sources

Yeast	Body site	Country of isolation	HIV status of patient	IC_{50} (mg/ml)		Source or reference
				Mean ± SD[a] (*n*)	Range	
C. dubliniensis						
P0508	Blood	Israel	−	0.043 ± 0.016 (6)	0.024–0.079	This study
CD2081	Oral cavity	Ireland	−	0.055 ± 0.018 (3)	0.040–0.082	This study
CD51-IIC[b]	Oral cavity	Ireland	+	0.057 ± 0.032 (6)	0.022–0.112	20
CD36[c]	Oral cavity	Ireland	+	0.072 ± 0.033 (3)	0.048–0.119	36
CM2[d]	Oral cavity	Australia	+	0.075 ± 0.015 (5)	0.054–0.099	20
CD2092	Oral	France	+	0.092 ± 0.036 (3)	0.050–0.140	This study
CD2093	Oral	France	+	0.095 ± 0.019 (3)	0.076–0.122	This study
CD411	Oral cavity	Ireland	+	0.103 ± 0.026 (3)	0.065–0.145	26
P8679	Retina	Israel	−	0.190 ± 0.060 (6)	0.130–0.079	This study
CBS8501	Blood	Holland	−	0.191 ± 0.115 (6)	0.088–0.440	19
P9224	Cornea	Israel	−	0.196 ± 0.022 (6)	0.170–0.230	This study
C. albicans 132A	Oral cavity	Ireland	+	0.096 ± 0.049 (6)	0.028–0.152	9

[a] Values shown are mean averages of several independent experiments (*n*) ± standard deviations. IC_{50} was determined as described in Materials and Methods.
[b] CD51-IIC is an in vitro-generated fluconazole-resistant derivative of *C. dubliniensis* clinical isolate CD51-II, which is fluconazole susceptible.
[c] CD36 is the *C. dubliniensis* type strain (36).
[d] CM2 is a fluconazole-resistant clinical isolate; all the other isolates tested were fluconazole susceptible.

only limited types of antifungal agents for patient treatment have created a need for new broad-spectrum, nontoxic antifungals, and therefore, the histatin peptide may be a promising candidate. While the susceptibility of *C. albicans* to histatin 5 and histatin 3 is well documented, the susceptibility of *C. dubliniensis* to histatin has not been studied. In this study we examined the susceptibility of 11 *C. dubliniensis* clinical isolates and derivatives to histatin 3 and found a range of susceptibilities to histatin 3 among the strains tested. The binding and internalization of histatin 3 in these organisms was also investigated to determine whether these factors might contribute to the altered susceptibilities found.

MATERIALS AND METHODS

Preparation of ^{14}C-labeled and fluorescein-labeled histatin. Histatin 3 (DSH AKR HHG YKR KFH EKH HSH RGY RSN YLY DN) was synthesized by Albachem, University of Edinburgh, Edinburgh, Scotland) and purified by high-performance liquid chromatography. The peptide was radiolabeled by reductive methylation using the method of Xu et al. (39). Radiolabeled histatin was stored in aliquots at −20°C. The specific activity of the [^{14}C]histatin was 5.36 Ci/mol. Fluorescent histatin 3 was made by Albachem using a 5,6-carboxyfluorescein conjugate at the N terminus of the peptide. It has been shown previously that the candidacidal activity of labeled histatin, either radiolabeled or fluorescently labeled, is not significantly affected by the labeling reaction (15, 39).

Candidacidal assay of histatin 3. The candidacidal assay used was a modification of the method previously described by Xu et al. (39). *C. dubliniensis* strains were grown on potato dextrose agar (PDA) (Oxoid, Dorset, United Kingdom) plates at 30°C for 48 h. A single colony was inoculated into 10 ml of yeast extract-peptone-dextrose (YEPD) medium and grown overnight at 30°C in a shaking incubator at 200 × rpm. Cells were washed with 0.9% (wt/vol) NaCl, counted using a hemocytometer, and resuspended at 10^6 cells/ml. Killing assays were performed in 100 μl of 10 mM potassium phosphate buffer, pH 7.4, and contained 10^5 cells and a range of histatin 3 concentrations (0 to 100 μM). The reaction was incubated for 1 h at 37°C with vigorous mixing. The mixture was then diluted 1/10 by adding 900 μl of 0.9% (wt/vol) NaCl, and 100 μl of each dilution was spread onto PDA plates, which was followed by incubation at 30°C for 48 h. The number of single colonies on each plate was then counted. Killing activity was calculated as the number of CFU on test plates as a percentage of CFU on control plates (cells incubated in the absence of histatin). The 50% inhibitory concentration (IC_{50}) determined was the concentration of histatin required to reduce the number of CFU by 50% and was determined visually from plots of killing activity versus histatin concentration.

Candidacidal assays were performed with 11 strains of *C. dubliniensis* and the *C. albicans* reference strain CA132A (Table 1). Candidacidal assays were performed on at least three separate occasions and the mean and standard errors were calculated for each strain.

Determination of K_d. Overnight cultures of *C. albicans* 132A or *C. dubliniensis* were grown from 48-h-old PDA plates in YEPD medium, and cell counts were estimated using a hemocytometer. Cultures were diluted to 2.5×10^8 cells/ml and washed, before resuspending in 250 μl of 10 mM potassium phosphate buffer. The reaction mixture contained 2×10^7 cells and a range of concentrations (0.5 to 20 μM) of ^{14}C-labeled histatin 3 (specific radioactivity, 5.36 Ci/mol) in a total volume of 200 μl. The mixture was then incubated at 37°C for 30 min with constant shaking (200 rpm). Following centrifugation (13,000 × *g*, 5 min, 4°C), cells were washed twice with phosphate-buffered saline and resuspended in 10 mM potassium phosphate buffer, pH 7.4. The histatin-bound pellet was transferred to a scintillation vial containing 4 ml of scintillation fluid (Ecoscint; National Diagnostics, Atlanta, Ga.) and counted in a liquid scintillation counter. Nonspecific binding was found to be negligible, as determined by adding a 10-fold excess of unlabeled histatin in the assay. Preliminary assays were performed to confirm that binding was linear with time. Binding assays were performed in duplicate on three separate occasions. The MACCURVEFIT software program, version 1.2, for Apple Macintosh computers (Kevin Raner Software, Mt. Waverley, Victoria, Australia) was used to determine the dissociation constant, K_d, from saturation curves using the equation $f(x) = (a \times x)/(b + x)$.

Visualization of histatin binding using fluorescein-labeled histatin 3. A modification of the method previously described by Xu et al. (39) was used for visualization of histatin binding. Freshly grown *C. albicans* or *C. dubliniensis* cells (3.4×10^6 cells/ml) were incubated with 12 nmol of carboxyfluorescein-labeled histatin in a total volume of 1 ml of potassium phosphate buffer, 10 mM, pH 7.4. Cells were incubated for 15, 45, and 90 min, centrifuged at 13,000 × *g* for 5 min, washed twice with 0.9% (wt/vol) NaCl and resuspended in 100 μl of distilled water. Cell suspensions were then spotted onto glass microscope slides, flame fixed quickly over a Bunsen burner, and air-dried in the dark. A drop of mounting medium (Sigma-Aldrich Chemical Company, Tallaght, Dublin, Ireland) was placed over the sample before covering with a glass coverslip. Slides were viewed under the 100× oil immersion lens of a Nikon (Eclipse ε600) microscope with a fluorescent light source (Nikon Super High Power Mercury Lamp). Fluorescent micrographs were taken at a set exposure time of 5 s.

RESULTS

Susceptibility testing of *C. dubliniensis* strains. The *C. dubliniensis* strains studied are listed in Table 1. The group included clinical isolates from HIV-infected and non-HIV-infected individuals, recovered from several body sites, representing various worldwide locations, and including fluconazole-resistant strains. Histatin 3 susceptibility curves for each strain tested are shown in Fig. 1. IC_{50}s were determined

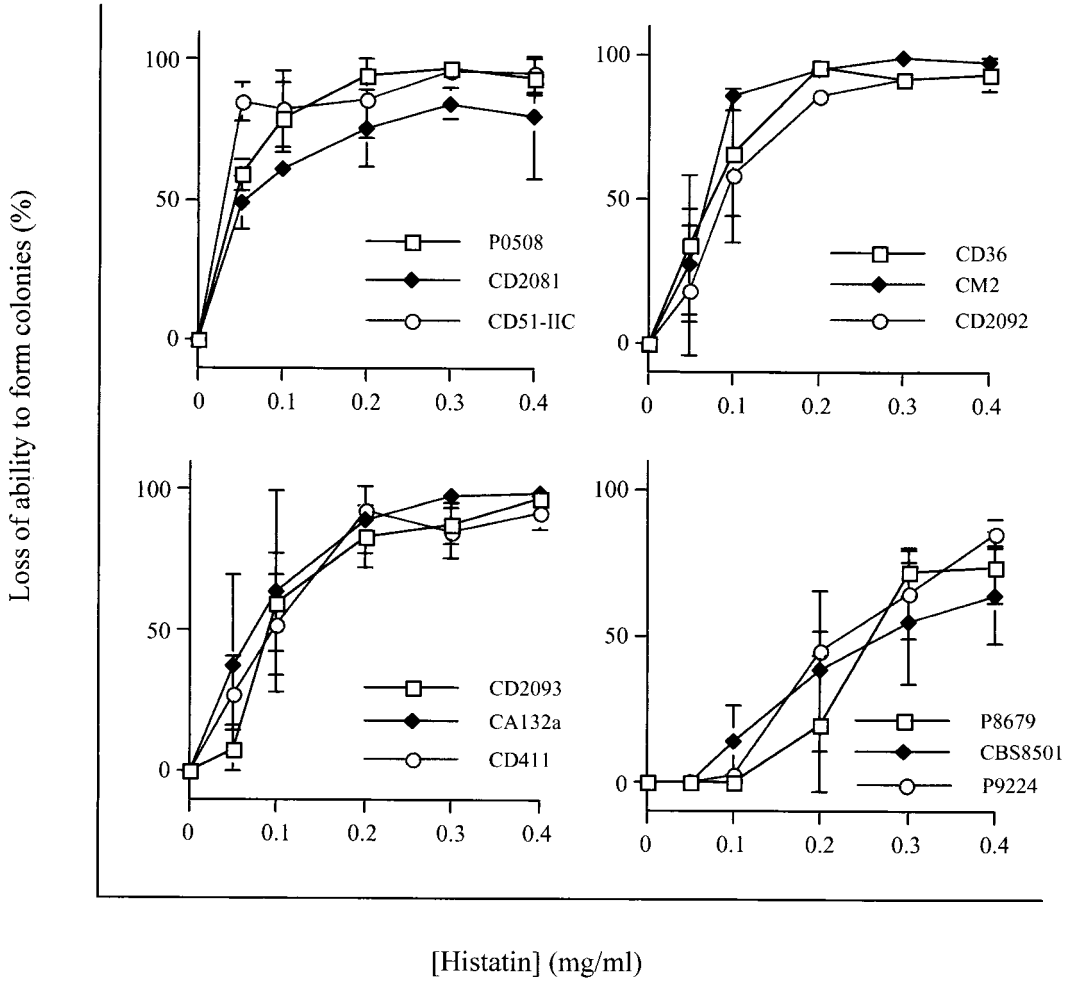

FIG. 1. Candidacidal activity of histatin 3 against various *C. dubliniensis* strains and *C. albicans* CA132A. Results are means ± standard deviations (error bars) of three independent assays. Where error bars are not visible, they are smaller than the data symbol.

from susceptibility curves and the values determined are shown in Table 1. All strains were susceptible to killing by histatin 3, but differential susceptibility to histatin 3 was found among the strains tested. The strains can be broadly divided into three categories based on the range of IC_{50}s found, namely, low-IC_{50}, high-IC_{50}, and intermediate-IC_{50} strains. Based on the small number of strains studied, it was not possible to correlate susceptibility to histatin 3 with any one global location or site of isolation. It is notable, however, that strains which showed decreased susceptibility to histatin 3 were from HIV-negative individuals, whereas the majority of those isolated from HIV-positive individuals fell into the low- or intermediate-IC_{50} category, which suggests that the histatins may have potential in the treatment of HIV-related candidal infection.

Since the antimicrobial activity of peptides may be influenced by the growth phase of the cells, we determined the affect of growth phase on the susceptibility to histatin 3 of *C. albicans* strain 132A and the most- and least-susceptible *C. dubliniensis* strains (*C. dubliniensis* P0508 and *C. dubliniensis* P9224). Cells in logarithmic (5 h, 37°C) and stationary (18 h, 37°C) phase of growth were compared for their susceptibilities to histatin, and the resulting IC_{50}s are shown in Table 2. The results show that, in each case, logarithmic-phase cells are more susceptible to histatin killing. However, the *C. dubliniensis* strain P9224 is the least susceptible to histatin in either phase of growth compared to *C. dubliniensis* P0508 or *C. albicans* 132A.

In *C. albicans* species, the binding of histatin 3 to a specific receptor on the fungal cell membrane is critical in the mechanism of killing, and it has been shown that binding is both temperature and salt concentration dependent (39). To inves-

TABLE 2. Comparison of histatin susceptibility of yeast cells grown to different phases

Yeast	Mean IC_{50} (mg/ml) ± SD	
	Logarithmic phase (n = 3)	Stationary phase (n = 6)
C. albicans 132A	0.014 ± 0.0004	0.096 ± 0.049
C. dubliniensis P0508	0.024 ± 0.011	0.043 ± 0.016
C. dubliniensis P9224	0.071 ± 0.017	0.196 ± 0.022

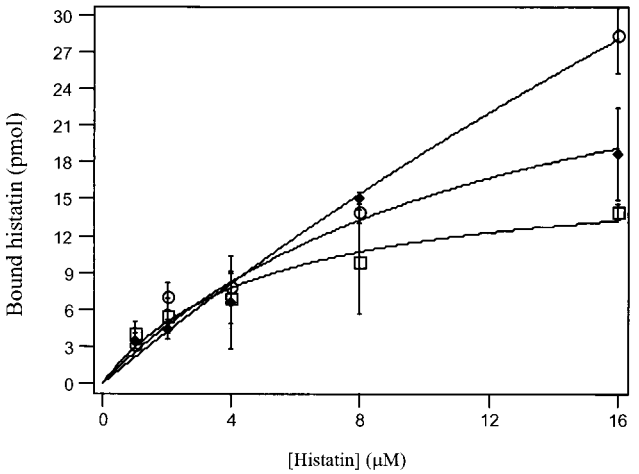

FIG. 2. Binding of ^{14}C-labeled histatin to CA132A (□), P0508 (◆), P9224 (○). A total of 10^7 cells/ml were incubated for 15 min with 0 to 16 μM [^{14}C]histatin 3. Assays were performed in 10 mM potassium phosphate buffer, pH 7.4. Data points shown are the means ± standard deviations (error bars) of duplicate determinations from a representative binding assay.

tigate whether alterations in the binding of histatin to its receptor might contribute to the susceptibility to histatin, binding assays were performed for the *C. dubliniensis* strain which gave the lowest IC_{50} (*C. dubliniensis* P0508; 0.043 mg/ml) and highest IC_{50} (*C. dubliniensis* P9224; 0.196 mg/ml) compared to the reference *C. albicans* (CA132A; 0.096 mg/ml), representing the intermediate IC_{50} range. The *C. albicans* reference strain CA132A was chosen as the control because the interaction of *C. albicans* with histatin has been extensively characterized and its susceptibility to histatin 3 (Table 1) and binding characteristics (data not shown) did not differ significantly from those of the *C. dubliniensis* reference strain CD36. Figure 2 shows the binding of [^{14}C]histatin 3 to cells of *C. albicans* CA132A and *C. dubliniensis* P0508 and P9224 as a function of the concentration of histatin in the assay medium. Both *C. albicans* CA132A and *C. dubliniensis* P0508 were shown to approach saturation at a concentration of about 12 μM histatin 3; however, the less-susceptible *C. dubliniensis* strain P9224 did not appear to saturate within the concentration range tested. The maximum concentration of [^{14}C]histatin that could be used in the assay was limited by the concentration of the stock solution. The dissociation constant (K_d) of histatin 3, at 37°C and pH 7.4, for each strain was determined from the binding plots using the MACCURVEFIT program for Apple Macintosh computers and were as follows (means ± standard deviations): *C. albicans* strain CA132A, 7.29 ± 2.04 μM; *C. dubliniensis* strain P0508, 21.1 ± 7.5 μM. Although *C. dubliniensis* strain P9224 did not reach saturation under these conditions, the estimated K_d was 67.5 ± 12.02 μM ($n = 3$).

Fluorescent histatin 3 was used to visualize the time course of histatin 3 internalization in the three *Candida* strains examined (*C. albicans* CA132A and *C. dubliniensis* P0508 and P9224). Cells from each strain were incubated with 12 μM fluorescent histatin for 15, 45, and 90 min, and the resulting micrographs are shown in Fig. 3. After 15 min of incubation histatin had accumulated in some cells of *C. albicans* CA132A and *C. dubliniensis* P0508, but *C. dubliniensis* P9224 exhibited significantly less internalization of histatin at that time point. After 45 min, accumulation of peptide in *C. albicans* CA132A and *C. dubliniensis* strain P0508 was observed in almost all cells, but in the case of *C. dubliniensis* P9224 only a small population (10% approximately) of cells had taken up the peptide. After 90 min of incubation with fluorescent histatin 3, the majority of *C. albicans* CA132A and *C. dubliniensis* P0508 cells were heavily stained, whereas the staining of *C. dubliniensis* P9224 cells was more patchy, with some cells appearing full of histatin, while others showed little uptake of the peptide. The binding and internalization pattern found here is typical of the pattern seen in *C. albicans* by other workers using fluorescein-labeled histatin 3 or histatin 5 (11, 15, 39). The characteristic initial binding of the labeled peptide to a discrete area of the cell membrane is clearly visible at the 15-min time point before it accumulates within the cell at the later time points. The morphology of the cells of *C. dubliniensis* P0508 differed from that of the two other strains tested. Cells appeared oval in shape rather than showing the rounded appearance of *C. albicans* CA132A and *C. dubliniensis* P9224 cells. Furthermore, at the 90-min time point, two types of cells could be distinguished in *C. dubliniensis* P0508, which significantly differed in their intensities of staining (lightly stained and heavily stained cells). This phenomenon was not apparent in *C. albicans* CA132A or *C. dubliniensis* P9224 cells (Fig. 3).

DISCUSSION

Over the last decade there has been an increase in the prevalence of fungal infections and in the range of infecting organisms, particularly in HIV-positive and immunocompromised patients (1, 30). However, there remains only a limited number of available antifungal agents. *C. dubliniensis* is a newly described *Candida* species which has a global distribution and is primarily associated with oral candidiasis in HIV-infected patients and patients with AIDS (36). While the majority of *C. dubliniensis* clinical isolates tested are susceptible to azole drugs such as fluconazole, ketoconazole, and itraconazole (14, 20, 25), azole-resistant clinical isolates have previously been reported (20). Therefore, there is now a need for new antifungal agents and a renewed interest in the use of host-derived antifungal agents, such as histatin.

Currently there are no reports in the literature on the susceptibility of *Candida dubliniensis* to histatin 3. We have shown in this study that histatin 3 is a potent inhibitor of *C. dubliniensis* and that within the *C. dubliniensis* species there is relatively wide variation in histatin susceptibility among different strains, ranging from IC_{50} 0.043 to 0.196 mg/ml. Importantly, the fluconazole-resistant *C. dubliniensis* clinical isolate CM2 and the in vitro-generated fluconazole-resistant *C. dubliniensis* derivative CD51-IIC were susceptible to histatin 3 killing (IC_{50} = 0.075 and 0.057 mg/ml, respectively). In both of these *C. dubliniensis* strains, fluconazole resistance has been associated with overexpression of the *MDR1* gene (20). This gene encodes the major facilitator protein Mdr1p, which plays a role in reducing the intracellular fluconazole concentration by a process of active drug efflux (20). The finding that these *C. dubliniensis* strains are susceptible to histatin, highlights the po-

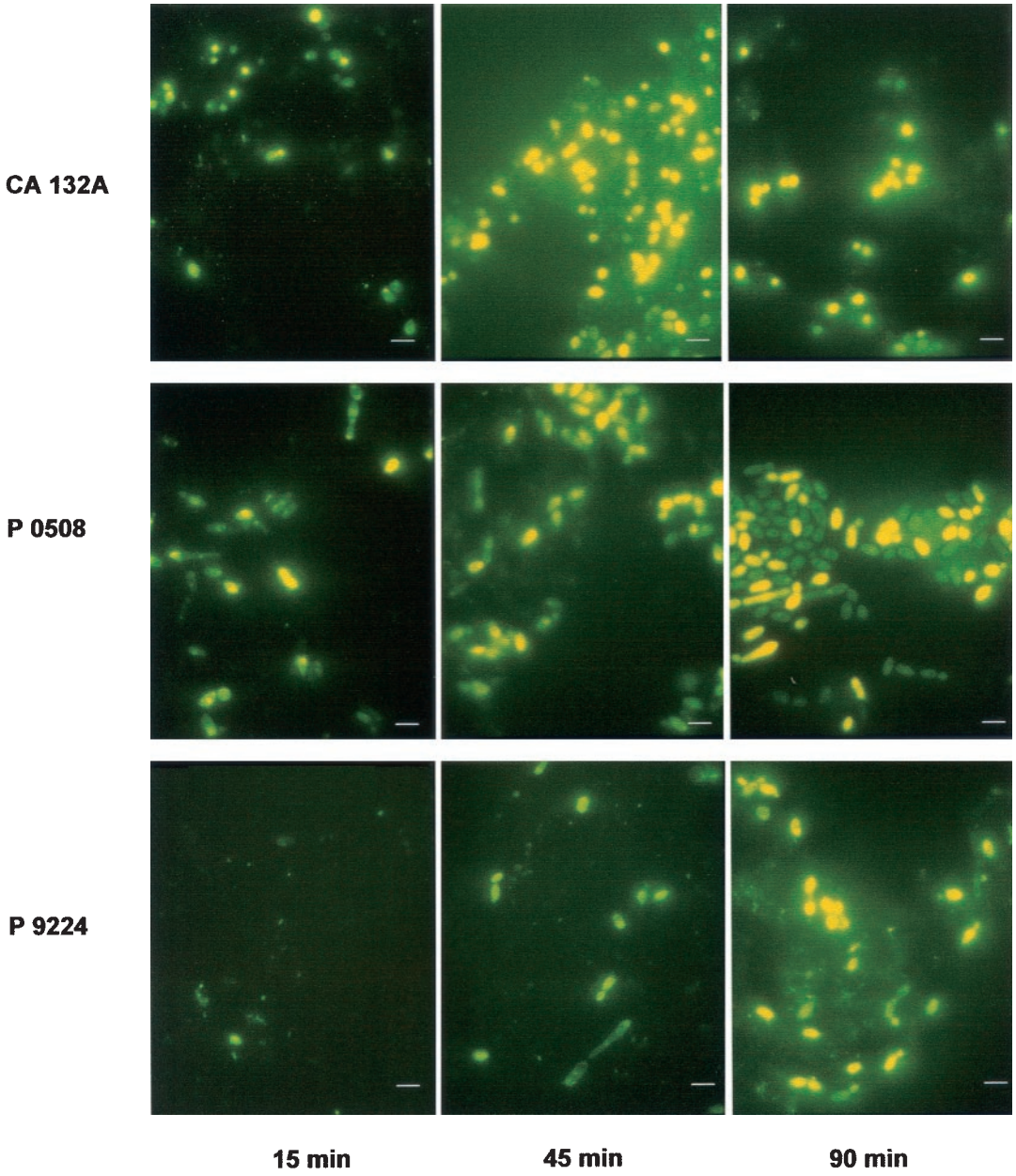

FIG. 3. Binding and internalization of fluorescently labeled histatin to *C. albicans* and *C. dubliniensis* strains. Cells from *C. albicans* CA132A and *C. dubliniensis* P0508 and P9224 were incubated with 12 μM fluorescently labeled histatin at 37°C for the periods indicated. The number of cells exposed in each case was 3.4×10^6 cells/ml. Micrographs are for a film exposure time of 5 s. Scale bars, 3 μm.

tential usefulness of histatin 3 as an effective alternative antifungal therapy.

Based on the small number of *C. dubliniensis* strains studied, it is difficult to correlate the variation in susceptibility to histatin with any particular anatomical site of isolation or global location or with the HIV status of the individuals from which the isolates were originally recovered. Recently, *C. dubliniensis* has been subdivided into four distinct genotypes based on comparative nucleotide sequence analysis of the internal transcribed spacer region of the ribosomal gene cluster and on DNA fingerprint analysis using the *C. dubliniensis*-specific Cd25 hybridization probe (10). The majority of genotype 1 isolates were recovered from HIV-infected individuals, whereas the majority of genotype 2 to 4 isolates were recovered from HIV-negative individuals ($P < 0.001$). It will be interesting to determine whether differences in histatin 3 susceptibility are associated with different genotypes.

The mechanism of action of histatin, though not yet fully elucidated, is different from that of the azole antifungal drugs, which disrupt ergosterol biosynthesis. In *C. albicans*, histatins exert their candidacidal activity by binding to a specific fungal membrane receptor (7), followed by internalization and interaction with intracellular targets, believed to be located in the mitochondria (11). Non-*C. albicans Candida* species including

C. glabrata, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, and *C. parapsilosis* have all been shown to be susceptible to histatin-mediated killing (23, 31). However, the mechanism of the antifungal activity of histatin has, to date, only been studied in *C. albicans*. The data presented in this study suggest that the mechanism of histatin 3-mediated killing in *C. dubliniensis* follows a similar mechanism involving the same targets, though confocal images would be useful to verify the fate of bound histatin 3. In support of this hypothesis, it has been shown that another yeast, *S. cerevisiae*, both is susceptible to histatin 5 and expresses a binding protein with a similar size to that in *C. albicans*, indicating that the antifungal mechanisms are similar (7). The variations in susceptibility of *C. dubliniensis* strains to histatin 3 found in the present study may reflect an alteration in binding to the histatin-binding protein, or in the interaction of histatin with cellular targets. In the present study, the higher dissociation constant (67.5 μM) determined for *C. dubliniensis* P9224 (the least-susceptible strain tested) suggests that histatin 3 has reduced affinity for this strain compared to the most susceptible *C. dubliniensis* P0508 (21.1 μM) or the reference *C. albicans* strain CA132A (7.29 μM). The reduced affinity of *C. dubliniensis* P9224 for histatin 3 may reflect a decrease in the number of binding sites or an alteration in the binding site itself in this isolate compared to the more susceptible isolates. The slower progress of histatin internalization in the *C. dubliniensis* strain P9224, compared to either the most-susceptible *C. dubliniensis* strain P0508 or the intermediately susceptible *C. albicans* strain CA132A, also suggests that there is a deficit in internalization of the peptide in *C. dubliniensis* P9224. Alternatively, less susceptible strains may exhibit differences in the expression of efflux pumps, similar to that described for fluconazole resistance in *C. albicans* and *C. dubliniensis* involving the ATP-binding cassette transporters Cdr1p and Cdr2p and the major facilitator protein Mdr1p (20, 33, 34). We cannot exclude the possibility that the histatin peptide is being pumped out of the cells of the less-susceptible *C. dubliniensis* strain P9224 by some unidentified transport system or is rapidly degraded at the surface. In this study, efflux of fluorescent peptide following incubation with fluorescent histatin could not be visualized as the cells were washed to remove unbound fluorescent histatin.

C. dubliniensis strain P0508 was found to be more susceptible to histatin 3 than the *C. albicans* reference strain CA132A (IC_{50} = 0.043 mg/ml, compared to 0.096 mg/ml). However, the increased susceptibility could not be attributed to greater binding affinity (K_d = 21.1 μM) (Fig. 2) or to increased rates of accumulation (Fig. 3). It is interesting that the cells of this strain showed two distinct staining intensities after 90 min of incubation with fluorescent histatin. It is possible that these two staining patterns represent different levels of expression of some yet unidentified protein, which is important in the accumulation or internal targeting of histatin. It is likely that at the 90-min time point a fully stained cell indicates that cell death has occurred and the cell has become fully saturated with the peptide. This pattern suggests that within *C. dubliniensis* strain P0508 there are individuals cells with different susceptibilities to histatin. Variations in fluconazole susceptibility have been previously reported among clonal isolates of *C. dubliniensis* recovered from the same oral specimen. In a recent study, by Gee et al. (10), of 15 clonal isolates recovered from the same clonal specimen from the oral cavity of an Irish AIDS patient, 4 isolates exhibited reduced susceptibility to fluconazole (MIC, 16 μg/ml) whereas the other 11 were fluconazole susceptible (MIC ≤ 1 μg/ml). Candidacidal assays which record loss of colony-forming cells would not necessarily reveal subpopulations of cells that had different susceptibilities to histatin 3. It would be important to investigate whether this variation in histatin internalization phenotype occurs in other susceptible *C. albicans* or *C. dubliniensis* strains, as this effect has not been reported previously in *C. albicans*.

The therapeutic potential of histatin is now becoming evident, particularly because the mechanism of its antifungal activity involves pathways distinct from that of the more commonly used azole drugs which affect ergosterol biosynthesis. The data presented here suggest that in *C. dubliniensis*, variations in susceptibility to histatin may involve alterations in the binding or internalization of histatin. The role of the specific histatin-binding protein in histatin-mediated killing of *Candida* species may be studied more directly by constructing knockout strains or by overexpressing the protein in mammalian cells, which are inherently resistant to histatin-mediated killing and in which the binding activity is undetectable (7). Identification and characterization of the specific histatin-binding protein and intracellular targets of histatin, which are now being undertaken in this laboratory, will be critical if the full potential of histatin as an antifungal is to be exploited. In this study we have shown that a number of *C. dubliniensis* isolates are susceptible to histatin 3 killing. It has been shown that histatin 5 kills many of the *Candida* species associated with oral candidiasis (37), and further characterization of the range of organisms susceptible to histatin 5 and histatin 3 killing is ongoing. In particular, it is important to ascertain whether the mechanism of histatin-mediated killing is the same for all susceptible *Candida* species or whether small differences in the pathway may affect histatin susceptibility.

This knowledge, together with a deeper understanding of the pathways involved in its mechanism of killing, may represent a way forward in the use of histatin-based drugs, alone or in addition to azole drugs, in the treatment of oral candidiasis and mucosal disease.

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