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Oral epithelium–*Candida glabrata* interactions *in vitro*

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Background: Oropharyngeal candidiasis is a common opportunistic infection and *Candida glabrata* is the second or third most frequently isolated species from oropharyngeal candidiasis lesions, after *Candida albicans*. The aim of this study was to study the cytokine-inducing and cell-damaging potential of *C. glabrata* in oral epithelial cells and compare this to *C. albicans*.
Methods: Oral epithelial cell lines and primary gingival epithelial cells were cocultured with *C. glabrata* strains GDH2269 and 94-11 or *C. albicans* strains SC5314 and ATCC28366. Supernatants were analysed for the presence of interleukin-1α (IL-1α), IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) by enzyme-linked immunosorbent assay. The cytotoxity of different strains was determined using the CytoTox-96 assay.
Results: Compared to *C. albicans*, *C. glabrata* induced different proinflammatory cytokine responses in oral epithelial cells; a high level of GM-CSF induction was only detected in *C. glabrata*-infected cells and not in *C. albicans*-infected cells, regardless of the origin of these cells (cell lines or primary cells) or the strain used. Like *C. albicans*, *C. glabrata* induced an IL-1α response by oral epithelial cells, but this response was both strain-dependent and epithelial cell origin-dependent. Unlike *C. albicans*, *C. glabrata* failed to induce a strong IL-8 response in any of the cell systems studied. Finally, in these studies *C. glabrata* showed lower cytotoxicity than *C. albicans*.
Conclusions: *C. glabrata* is less cytotoxic than *C. albicans* and induces different proinflammatory cytokine responses in oral epithelial cells.

Key words: *Candida glabrata*; cytokine; cytotoxicity; oral epithelial cell

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Oral candidiasis is a common opportunistic infection in immunocompromised populations (27). *Candida albicans* is the most prevalent and best studied etiological agent associated with this infection, accounting for 70–80% of organisms isolated form oral mucosal lesions (26, 28). However, in the past two decades, *Candida glabrata* has emerged as a notable pathogenic agent in the oral mucosa, frequently being co-isolated with *C. albicans* (24–26). Meanwhile, reports of *C. glabrata* being the only detectable species from oral lesions have also been rising steadily (24–26). This is particularly important because *C. glabrata* isolated from oral lesions is much more resistant to standard antifungal treatment than *C. albicans* (24, 26). As a result, *C. glabrata* oral infection is suspected in most cases when the patient does not respond to routine doses of fluconazole (25).

Oral epithelial cells play several important roles in the host defense against *Candida* infection. Integrity of the epithelial layer is required for the confinement of *C. albicans* in the superficial mucosal layers because removal of the epithelium before experimental yeast inoculation leads to rapid connective tissue invasion (14). In addition to maintaining an intact invasion barrier, oral epithelial cells can regulate the inflammatory host responses to *Candida* by releasing a wide array of chemotactic (8, 30, 31) and priming (10, 25, 35) molecules for innate immune effector cells. As the best studied *Candida* species, *C. albicans* is a potent inducer of several immunomodulatory cytokines, such as interleukin-1α (IL-1α) and IL-8, in human oral keratinocytes (7, 27, 32). Furthermore, the generation of these cytokines by *C. albicans*-infected oral epithelial cells promotes the antifungal activity of polymorphonuclear leukocytes *in vitro*, which are among the most important immune effector cells in the host defense against *Candida* infection (10).

Although *C. glabrata* has been implicated in the pathogenesis of oral candidiasis in several categories of immunosuppressed patient, little is known about the outcome of the interaction between *C. glabrata* and

oral mucosal cells *in vitro*. Given the scarcity of information in the field, we sought to study the cytokine-inducing and cytotoxic potential of *C. glabrata* using a *C. glabrata*–oral epithelial cell coculture *in vitro* system, and to compare it to *C. albicans*.

Materials and methods

Organisms

C. glabrata strains GDH2269 and 94-11 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Both strains were isolated from human oral cavities. *C. glabrata* strain MRL2302 was isolated from a patient with esophageal candidiasis and was kindly provided by Dr M. Ghannoum (Case Western Reserve University, Department of Dermatology). Two *C. albicans* strains were used for comparison: *C. albicans* strain SC5314 (kindly provided by Dr A. Mitchell, Columbia University), which was originally isolated from a patient with disseminated candidiasis (13) and has the ability to trigger high levels of proinflammatory cytokines and cell damage (8, 40); and *C. albicans* oral strain ATCC28366, which exhibits a moderate ability to do trigger these in oral mucosal models *in vitro* (9, 41). All of the strains used in this study showed similar growth rates in the inoculation media used for infection, as determined by direct cell counts of yeast cells grown in these media, or by the 2,3-bis(2-methoxy-4-nitro-5-sulfopheny)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT) assay when germinated organisms were tested. The organisms were routinely propagated in Yeast Peptone Dextrose or Sabouraud agar (Difco Laboratorie, Detroit, MI), at 25°C.

Oral epithelial cell cultures

Oral keratinocyte cell lines and primary gingival keratinocytes were used in this study. Cell line SCC15, originated from a well-differentiated squamous cell carcinoma of the ventral tongue, was kindly provided by Dr D. Wong (Harvard University). Cell line OKF6/TERT2 represents normal oral mucosal epithelium and was immortalized by overexpression of telomerase and deletion of the p16INK4a regulatory protein (6). Primary oral keratinocytes were isolated from discarded gingival tissues as described previously (8, 9). Keratinocytes were maintained in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA), supplemented with 0.4 m M $CaCl_2$, 0.1 ng/ml epidermal growth factor, 50 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA) and antibiotics (penicillin/streptomycin, 100 U/ml and 100 μg/ml, respectively).

Coculture of *C. glabrata* and *C. albicans* with oral epithelial cells

Stationary-phase yeasts were prepared by growth for 18 h at room temperature in yeast peptone dextrose broth (strains GDH2269, 94-11, ATCC2269 and SC5314) or Sabouraud broth (strains GDH2269 and MRL2302) (Difco Laboratories, Detroit, MI), supplemented with 2% (weight/volume) glucose. In preliminary experiments, *C. glabrata* strains GDH2269 and MRL2302 were tested with both types of broth and no difference was observed in growth kinetics or cytokine induction. The fungal cells were harvested by centrifugation and washed in phosphate-buffered saline. Subsequently, yeast cells were counted in a hemocytometer and adjusted to the final concentration in complete keratinocyte serum-free medium before adding to epithelial cells.

Oral keratinocytes were seeded at or near confluence in 24-well polystyrene plates (Corning Incorporated, Corning, NY) and were incubated overnight in complete keratinocyte serum-free medium at 37°C in 5% CO_2. The following day the media were discarded and the cells were challenged with suspensions of stationary-phase viable organisms at varying fungal cell to host cell ratios, for up to 48 h. Negative controls for these experiments included uninfected cultures and *Candida* alone. At the end of the experimental period supernatants were collected and stored at −70°C until assayed.

Cytokine detection

Cytokine protein array

Proinflammatory cytokines in culture supernatants were detected using an enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array (Ray Bio cytokine array; RayBiotech, Norcross, GA). Briefly, after membrane blocking, 1 ml supernatant was added and incubated for 2 h, followed by addition of biotinylated detection antibodies at a dilution of 4 : 1000. The membranes were developed by addition of horseradish peroxidase-conjugated streptavidin for 2 h and subsequent addition of an enhanced chemiluminescence-type solution. The membranes were exposed to X-ray film (Kodak BioMax film; Kodak, Rochester, NY) for 1 min and processed by autoradiography.

Enzyme-linked immunosorbent assay

To confirm the findings with cytokine arrays, culture supernatants were analysed by ELISA. In each experiment supernatants from two replicate wells were pooled and assayed by duplicate sandwich ELISAs using commercially available antibody pairs (Endogen MiniKit, Pierce, Rockford, IL), as previously described (7–9). Absorbance values and corresponding cytokine concentrations were determined with an Opsys MR Microplate reader (Dynex Technologies Inc., Chantilly, VA) using the Revelation QuickLink® software (Thermo Labsystems, Chantilly, VA). The sensitivity of these assays ranged between 8 and 16 pg/ml.

Assessment of cytotoxicity

The ability of *Candida* to injure oral epithelial cells was assessed by the CytoTox-96® assay (Promega, Madison, WI), which measures the release of lactate dehydrogenase (LDH) from dying cells. Total LDH released was quantified by spectrophotometry, as described previously (9). Spontaneous release of LDH by uninfected cultures, or by *Candida* alone, incubated under identical conditions was included as a negative control in each experiment.

Statistical analyses

The statistical significance of the differences in cytokine levels and the cytotoxicity between pairs of different *Candida* species were determined by two-tailed *t*-test, assuming equal variances. Differences were statistically significant at $P < 0.05$.

Results

C. glabrata induced a proinflammatory cytokine response by oral epithelial cells

To determine whether coculture of oral epithelial cells with *C. glabrata* induces the production of proinflammatory cytokines, we infected epithelial cells with various doses of yeast (ratios of 0.1, 1 and 10 yeast cells to epithelial cells) for up to 48 h. The presence of IL-1α, IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) in culture supernatants was quantified by ELISA and verified by a cytokine protein array. As shown in Fig. 1, *C. glabrata* induced a dose-dependent IL-1α response in SCC15 cells.

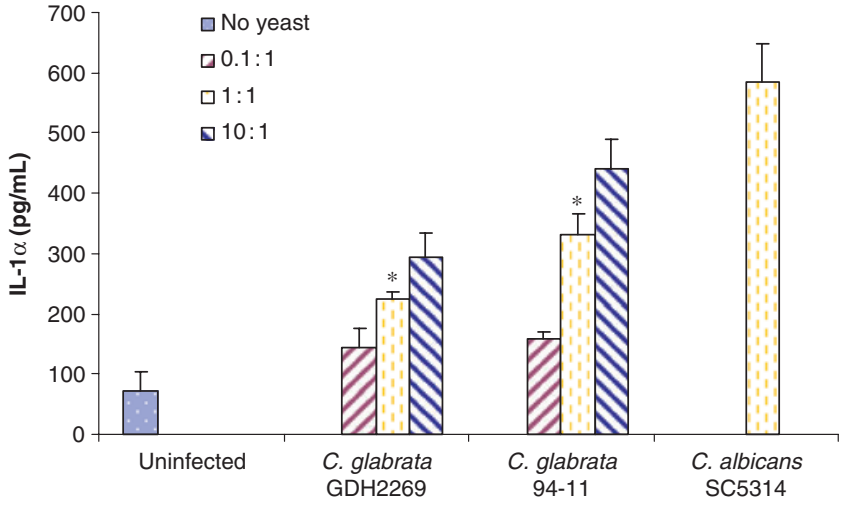

Fig. 1. Interleukin-1α (IL-1α) response of oral epithelial cell line SCC15 to *Candida glabrata*. Cells were challenged with *C. glabrata* strains GDH2269 or 94-11 or *Candida albicans* SC5314 at fungal cell to epithelial cell ratios of 0.1 : 1, 1 : 1 and 10 : 1. The presence of IL-1α was detected 36 h postinfection by ELISA. Mean values were obtained by analysis of three individual experiments with each condition set up in triplicate. The bars represent one SEM of the mean values. *$P < 0.05$ for a comparison with uninfected cells.

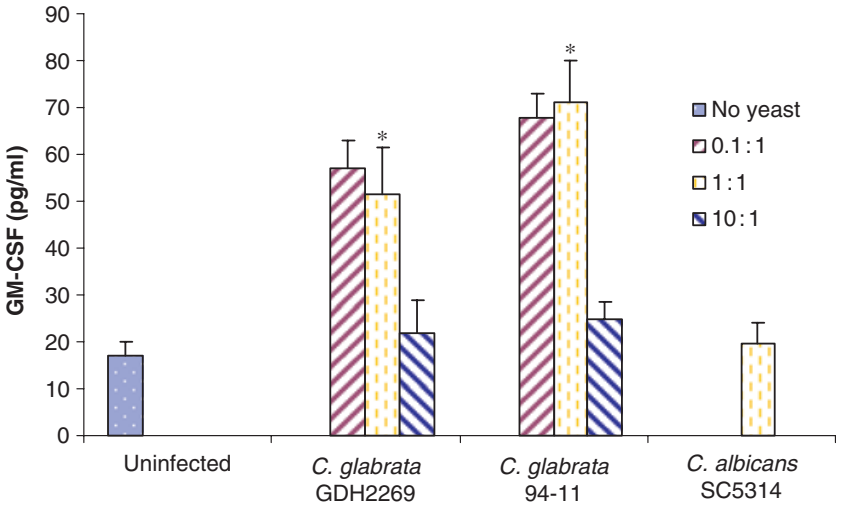

Fig. 2. Granulocyte–macrophage colony stimulating factor (GM-CSF) response of oral epithelial cell line SCC15 to *Candida glabrata*. Cells were challenged with *C. glabrata* strains GDH2269 or 94-11 or *Candida albicans* SC5314 at fungal cell to epithelial cell ratios of 0.1 : 1, 1 : 1 and 10 : 1. The presence of GM-CSF was detected 36 h postinfection by ELISA. Mean values were obtained by analysis of three individual experiments with each condition set up in triplicate. The bars represent one SEM of the mean values. *$P < 0.05$ for a comparison with uninfected cells.

It also triggered a significant GM-CSF response at lower infectivity doses (0.1 : 1 and 1 : 1), whereas the highest dose (10 : 1) was not stimulatory (Fig. 2). Furthermore, regardless of the infectivity ratio, neither *C. glabrata* strain induced an IL-8 response in SCC15 cells (Fig. 3).

In additional experiments, OKF6/TERT2 cells were infected by *C. glabrata* GDH2269 or MRL2303 for 24 h, and the cytokine responses of these cells were studied. The OKF6/TERT2 cells responded to strain GDH2269 in a similar manner to the SCC15 cells, by up-regulating the production of IL-1α and GM-CSF (Fig. 4B). However, strain MRL2302 showed a slightly different profile from the other two *C. glabrata* strains because it did not induce an IL-1α response (Fig. 4C). In addition, up-regulated production of growth-related oncogene was observed in cells infected by this strain. These results were confirmed by ELISA (not shown).

We have previously reported that the proinflammatory cytokine responses of SCC15 and OKF6/TERT2 cells to *C. albicans* closely resemble those of primary cells (8, 9). However, there is, as yet, no report on the cytokine responses of primary oral keratinocytes to *C. glabrata*. Therefore we compared the proinflammatory cytokine responses of these cell lines with primary gingival epithelial cells. As a result of the limited life span of these cultures *in vitro*, data from a single experiment with multiple cell cultures are shown in Table 1. Coculture of primary gingival keratinocytes with *C. glabrata* did not induce a strong IL-1α or IL-8 response (more than two-fold over basal). However, consistent with cell lines, primary gingival keratinocytes showed a strong GM-CSF response to infection with *C. glabrata* (at least two-fold over basal) at the lowest infectivity ratio (Table 1).

The *C. glabrata*-induced proinflammatory cytokine profile in oral epithelial cells differs from that of *C. albicans*

We and others have previously reported that *C. albicans* triggered a strong IL-1α and IL-8, but a weak, strain-dependent GM-CSF response in oral epithelial cells (7, 32). To compare the cytokine-inducing potential of *C. glabrata* with that of *C. albicans*, SCC15 cells were cocultured with *C. albicans* strain SC5314 at an infectivity ratio of 1 : 1 for up to 36 h. The 1 : 1 yeast to epithelial cell ratio was previously documented as the optimal infectivity ratio for *C. albicans* stimulation of both IL-1α and IL-8 in oral keratinocytes (8, 9). Consistent with prior findings, *C. albicans* SC5314 triggered a strong IL-1α and IL-8 response, but a weak GM-CSF response in SCC15 cells and primary keratinocytes. When compared with *C. albicans*, *C. glabrata* induced a lower IL-1α and IL-8 response (Figs 1 and 3 and Table 1). Interestingly, the GM-CSF response of SCC15 cells and primary keratinocytes to both *C. glabrata* strains was significantly higher than that to *C. albicans* SC5314 after 36 h of infection (Fig. 2 and Table 1).

C. glabrata was less cytotoxic to oral epithelial cells compared to *C. albicans*

We next characterized the cell-damaging potential of the two oral *C. glabrata* strains in comparison to *C. albicans* strain SC5314. The cytotoxicity of these organisms was tested in monolayer cultures of SCC15 and primary cells by measuring

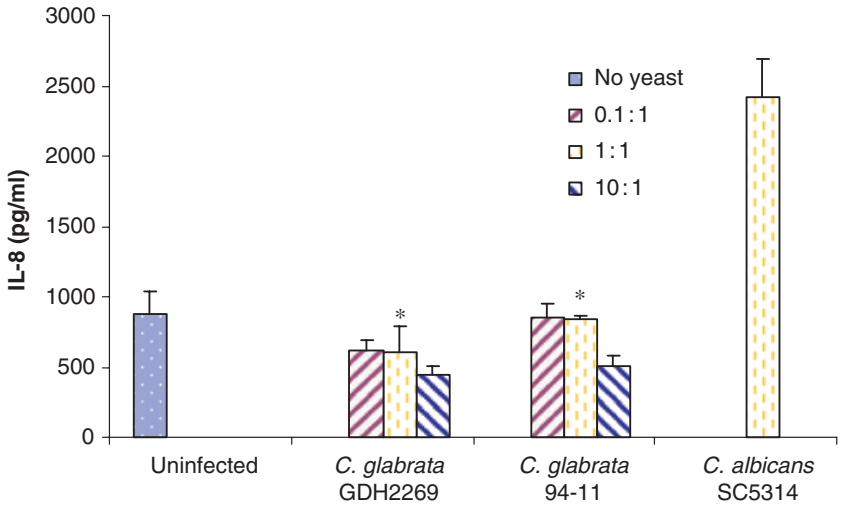

Fig. 3. Interleukin-8 (IL-8) response of oral epithelial cell line SCC15 to *Candida glabrata*. Cells were challenged with *C. glabrata* strains GDH2269 or 94-11 or *Candida albicans* SC5314 at fungal cell to epithelial cell ratios of 0.1 : 1, 1 : 1 and 10 : 1. The presence of IL-8 was detected 36 h postinfection by ELISA. Mean values were obtained by analysis of three individual experiments with each condition set up in triplicate. The bars represent one SEM of the mean values. *$P < 0.05$ for a comparison with uninfected cells.

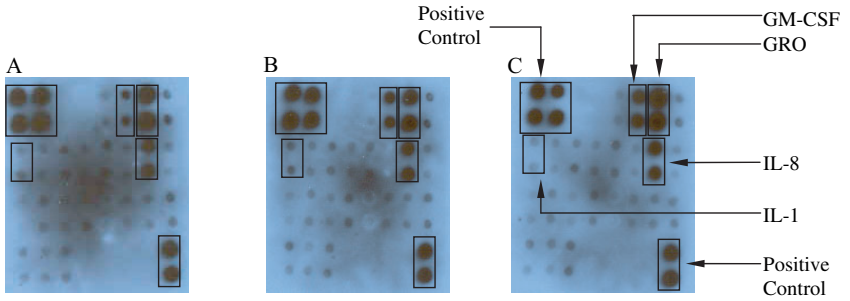

Fig. 4. Candida glabrata-induced cytokine induction in OKF6/TERT2 cells. Cells were uninfected (A), or infected with *C. glabrata* strain GDH2269 (B) or *C. glabrata* strain MRL2302 (C) at a 1 : 1 fungal cell to epithelial cell ratio for 24 h. Cytokine profiles in culture supernatants were detected using a human cytokine protein array.

LDH released in culture supernatants during infection. *C. albicans* strain SC5314 has been shown by our group and others to be cytotoxic in several cell culture systems (9, 12). Consistent with earlier findings, *C. albicans* SC5314 demonstrated a high level of cytotoxicity because the LDH released in the presence of these organisms was significantly higher ($P < 0.05$) than the spontaneous release 24 h postinfection (Table 2). Compared with *C. albicans*, the cytotoxic potential of the two *C. glabrata* strains 94-11 and GDH2269 was much lower ($P < 0.05$). In fact, even after 24 h of infection, the LDH released in the presence of *C. glabrata* was not significantly higher than that of the uninfected control ($P = 0.16$) (Table 2).

To verify that the cytotoxic potential of *C. glabrata* was not limited to transformed epithelial cell lines, we also compared the cytotoxic effect of *C. glabrata* and *C. albicans* using primary gingival epithelial cell cultures from two different human donors. As shown in Table 2, *C. glabrata* strains 94-11 and GDH2269 exerted a similar degree of cytotoxicity in primary cells compared to SCC15 cells, which was lower than that exerted by *C. albicans* strains (Table 2).

Discussion

Candida species are the most common opportunistic fungal pathogens in humans, with *C. albicans* being the most prevalent pathogen in mucosal and systemic fungal infections (23, 36). Historically, *C. glabrata* has been considered a non-pathogenic saprophyte of the normal flora in healthy individuals (33). However, in the past two decades, as a consequence of the widespread use of immunosuppressive therapy and broad-spectrum antimycotic prophylaxis, *C. glabrata* has emerged as an important opportunistic oral pathogen, ranking as the second or third most frequently isolated *Candida* species from oral candidiasis (11). Although the pathogenicity of *C. albicans* in the oral mucosa has been well established (4, 7), little is known about the outcome of the interaction between *C. glabrata* and oral mucosal cells. So far there is only one other report of the oral mucosal cytokine responses to *C. glabrata* using a cell-line-based reconstituted human epithelial model of infection with a single strain of *C. glabrata* (31).

A significant finding of our study is the ability of *C. glabrata* to trigger GM-CSF secretion in oral epithelial cells, which is significantly higher than that of *C. albicans*, regardless of the cell system studied. GM-CSF may be an essential proinflammatory cytokine in the protection against oral fungal infection and subsequent stromal invasion because it may promote the growth and local activation of innate immune effector cells, such as neutrophils and monocytes (5, 20). It has the ability to augment the fungicidal activities of these immunoeffector cells (2, 19). Clinically, administration of this cytokine as an adjunctive treatment of fluconazole-refractory oropharyngeal candidasis in patients with acquired immunodeficiency syndrome led to a significant beneficial effect on the oral mycoflora and helped clear the infection (39).

Although an increased release of GM-CSF by *C. glabrata*-infected oral epithelial cells has been observed by us and others (31), the mechanism and signaling pathways responsible for induction of this crucial pro-inflammatory cytokine are unknown. Toll-like receptors have been demonstrated to be important for recognition of fungal pathogens and activation of innate immune responses (16, 18). *In vitro*, *Candida* phospholipomannan has been proposed as the toll-like receptor 2 ligand, which induces the release of anti-inflammatory cytokines by J774 mouse cells (16). In contrast, binding of *Candida* mannan to toll-like receptor 4 led to the release of proinflammatory cytokines and chemokines by human monocytes (34). In human gingival epithelial cells, the expression of toll-like receptors 2 and 4 at the mRNA level has been identified (38). Interestingly, up-regulation of their expression in human gingival epithelial cells led

Table 1. Proinflammatory cytokine responses of primary oral keratinocytes to *Candida glabrata*

	Cytokine level in the culture supernatant (pg/ml)							
		C. glabrata GDH2269			*C. glabrata* 94-11			*C. albicans* SC5314
Culture #	Basal	0.1 : 1	1 : 1	10 : 1	0.1 : 1	1 : 1	10 : 1	1 : 1
IL-1α								
61#	928	969	867	813	842	867	701	2264
63#	345	312	374	442	279	353	423	3899
67#	291	219	208	260	302	320	236	689
IL-8								
61#	2075	2356	1761	1723	2975	1865	1621	4759
63#	2408	3853	2637	2406	6611	4855	5020	4355
67#	1541	3173	1102	1200	3590	2498	1744	3711
GM-CSF								
61#	181	340	155	171	352	155	148	176
63#	152	395	347	278	503	420	297	250
67#	51	355	106	101	349	191	126	23

IL-1α, interleukin-1α; IL-8, interleukin-8; GM-CSF, granulocyte–macrophage colony stimulating factor.

Primary gingival keratinocytes from three individual gingival tissue donors (passage 2 or 3) were cultured in the presence or absence (control) of live *C. glabrata* added at ratios of 0.1 : 1, 1 : 1 or 10 : 1 yeast cells to oral epithelial cells. *Candida albicans* was used at 1 : 1 yeast cell to oral epithelial cell ratio as a positive control. The presence of cytokines in the supernatant was detected 36 h postinfection by ELISA.

Table 2. Comparison of cytotoxic potential of *Candida glabrata* and *Candida albicans*

Cells	*C. glabrata* GDH2269	*C. glabrata* 94-11	*C. albicans* ATCC28366	*C. albicans* SC5314
83#	1.279	1.326	1.927	2.017
94#	1.486	1.582	2.942	3.112
SCC15	1.311 ± 0.170*	1.794 ± 0.246*	3.569 ± 0.214	4.582 ± 0.361

Primary gingival keratinocytes from two individual gingival tissue donors (passage 3) and the oral epithelial cell line SCC15 were cocultured with *C. glabrata* or *C. albicans* for 24 h, at a ratio of 1 : 1 yeast cells to epithelial cells. Cell supernatants were analysed for the presence of LDH (fold increase over uninfected). Data from SCC15 cells represent the average of triplicate wells from two independent experiments. Data from a single experiment with two individual primary cultures are also shown.

*$P < 0.05$ for a comparison with strain SC5314.

to enhanced GM-CSF synthesis upon stimulation of various bacterial components (37). Therefore it is highly likely that toll-like receptors 2 and/or 4 are involved in the signaling pathway responsible for GM-CSF up-regulation in *C. glabrata*-infected oral epithelial cells.

Using oral epithelial cell lines provides a number of benefits in the study of pathogen–host interactions, which include cost efficiency, practical simplicity and better reproducibility as a result of the lack of donor-to-donor variations. In this study we showed that cell lines differ from primary cells in their cytokine responses to *C. glabrata*. A discrepancy in the proinflammatory cytokine responses between primary cells and immortalized cell lines to the same stimulus has been reported by other groups (21, 22). For example, primary airway epithelial cells differ from cell line A549 in the tumor necrosis factor-α-induced IL-8 synthesis (22). In addition, differences in cytokine induction among different cell lines from the same tissue origin have been reported (38). The different cytokine up-regulation capacity in primary keratinocytes and cell lines may reflect the different constitutive expression levels of certain receptors on the cell surface, such as CD14 (38) and toll-like receptors (18).

Compared with *C. albicans*, *C. glabrata* demonstrated significantly lower cytotoxicity in our *C. glabrata*–oral epithelial cell coculture system. The higher potential of *C. albicans* to injure oral epithelial cells may be the result of several virulence factors, including protease production and phospholipase secretion. Secreted aspartyl proteases, the best-studied group of hydrolytic enzymes produced by *C. albicans*, contribute to cell damage and tissue invasion by damaging host cell membranes (15). Similarly, expression of phospholipase on the hyphal tips of *C. albicans* allows this organism to enter the cytoplasm of host cells by digesting the cell membrane (1). In contrast to *C. albicans*, *C. glabrata* failed to produce significant levels of protease activity *in vitro* (3). Furthermore, the production of phospholipases by *C. glabrata* was only detected in a small fraction of clinical isolates, and at a significantly lower level of production compared to the co-isolated *C. albicans* strains (17, 29). The lower level of production of extracellular hydrolytic enzymes in *C. glabrata* may be responsible for the low cytotoxicity of this organism.

Taken together, our findings showed that *C. albicans* and *C. glabrata* have significant differences in the proinflammatory cytokine profiles triggered in oral epithelial cells, with *C. glabrata* triggering a more pronounced GM-CSF and *C. albicans* triggering a more pronounced IL-8 and IL-1α response. In addition, compared with *C. albicans*, *C. glabrata* had lower cytotoxic potential. The different cytokine profile induced by *C. glabrata* and *C. albicans* suggests that distinct strategies are utilized by human oral epithelial cells to mediate mucosal inflammation and protection from different species. The low cell-damaging potential of the two *C. glabrata* strains tested in this study questions their potential for direct participation in mucosal damage during infection. Future studies using a larger number of lesion-associated *C. glabrata* strains are needed to clarify the role of this organism in mucosal destruction.

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