Candida glabrata colonizes but does not often disseminate from the mouse caecum

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Candida glabrata is the second or third most frequent cause of candidaemia. The gastrointestinal tract is considered to be a major portal of entry for systemic candidiasis, but relatively few studies have investigated the pathogenesis of *C. glabrata*. Experiments were designed to clarify the ability of *C. glabrata* to disseminate from the mouse intestinal tract. Following oral inoculation, *C. glabrata* readily colonized the caeca [approx. 10^7 cells (g caecum)⁻¹] of antibiotic-treated mice, but extraintestinal dissemination was not detected. Superimposing several mouse models of trauma and/or immunosuppression known to induce dissemination of *Candida albicans* and other intestinal microbes did not cause *C. glabrata* to disseminate often, although one exception was mice given high doses of dexamethasone for 4 days. These data support the hypothesis that the antibiotic-treated mouse intestine may be an epidemiological reservoir for *C. glabrata* and that this yeast tends to disseminate under specific clinical conditions.

Received 2 November 2006

Accepted 30 January 2007

INTRODUCTION

Systemic fungal infections are a major cause of morbidity and mortality in the critically ill, and a large proportion of infections occur in critical care units, including neonatal intensive care units (Rangel-Frausto *et al.*, 1999). *Candida* species are the fourth leading cause of nosocomial bloodstream infections (Rangel-Frausto *et al.*, 1999; Pappas *et al.*, 2003; Wisplinghoff *et al.*, 2004). Crude and attributable mortality are estimated at 57 and 38 %, respectively (Ruhnke, 2006). Risk factors for systemic candidiasis include prolonged antimicrobial therapy, glucocorticosteroids, peritoneal dialysis, haemodialysis, chemotherapy, radiation therapy, immunosuppression, mechanical ventilation, parenteral nutrition, high fungal colonization, neutropenia, bladder catheterization, intravascular catheters, complicated abdominal surgery, length of stay in the intensive care unit, and prematurity or very low birth weight (Charles *et al.*, 2003; Pappas *et al.*, 2003; Peres-Bota *et al.*, 2004; Wisplinghoff *et al.*, 2004). *Candida albicans* has historically accounted for the majority of *Candida* isolates, but non-*albicans Candida* are increasing in prevalence and *Candida glabrata* is now the second or third most frequently isolated *Candida* species (Rangel-Frausto *et al.*, 1999; Pappas *et al.*, 2003; Peres-Bota *et al.*, 2004;

Wisplinghoff *et al.*, 2004). Interestingly, of the more than 80 known *Candida* species, only *C. glabrata* exists solely as budding yeast and does not readily form hyphal elements, except under *in vitro* conditions of limiting nitrogen (Csank & Haynes, 2000).

The gastrointestinal tract is believed to be the major colonizing habitat and a major source of systemic candidiasis (Cole *et al.*, 1996; Blijlevens *et al.*, 2002). Experimental studies provide direct evidence that *C. albicans* can disseminate from the intestinal lumen to extraintestinal sites. For example, extraintestinal dissemination of *C. albicans* has been reported in immunosuppressed gnotobiotic piglets (Andrulis *et al.*, 2000) and in rodents given either broad-spectrum oral antibiotics (Kennedy & Volz, 1985; Berg *et al.*, 1993) or oral antibiotics coupled with immunosuppression (Ekenna & Sherertz, 1987; Bendel *et al.*, 2002, 2003). Using several strains of congenitally immunodeficient germ-free mice, Cantorna & Balish (1990) reported some systemic spread after oral inoculation of *C. albicans*, although progressive systemic infection was seen only in mice with severe combined immunodeficiency. Using a severe model, Alexander *et al.* (1990) observed *C. albicans* within the intestinal epithelium and lamina propria of thermally injured guinea pigs following direct injection of yeast into the intestinal lumen. Few studies have focused on *C. glabrata* and relatively little is known about its pathogenesis (Fidel *et al.*, 1999; Ruhnke, 2006). Our goal was to develop

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Abbreviations: i.p., intraperitoneally; MLNs, mesenteric lymph nodes.

a mouse model to study the pathogenesis of systemic *C. glabrata* acquired via the intestinal route. The results were surprising and indicated that although *C. glabrata* readily colonized the mouse intestine, extraintestinal invasion did not often occur.

METHODS

Micro-organisms and mice. *C. glabrata* ATCC 15126 was obtained from the ATCC. *Escherichia coli* M21 is a streptomycin-resistant rodent isolate. For experiments, overnight cultures were washed and diluted in Hanks' balanced salts solution. Microbial concentrations were determined using a haemocytometer (*C. glabrata*) or by densitometry (*Escherichia coli*) and confirmed by quantitative culture on appropriate agar media. Female 18–22 g Swiss Webster mice (Harlan Sprague–Dawley) were allowed to acclimatize for 1 week before experiments. Male mice were not used because they tend to fight and wound each other, confounding the origin of systemic infection. Mice were killed by cervical dislocation. Experiments were performed according to the National Institutes of Health guidelines, and the University of Minnesota Institutional Animal Care and Use Committee approved all protocols.

Mouse model of systemic candidiasis. The classical model of intravenous injection of yeast cells into the tail vein (Spellberg *et al.*, 2005) was used to assess the systemic virulence of this *C. glabrata* strain. Mice were injected with 0.1 ml containing a defined number of yeast. In a preliminary experiment, 40% mortality was noted after injection of 10^9 *C. glabrata* cells, with no mortality after injection of 10^8 or 10^7 cells. In subsequent experiments, mice were injected with 5×10^7 *C. glabrata* and randomly chosen to be sacrificed 1, 8 and 15 days later for quantitative culture of kidney, liver and mesenteric lymph nodes (MLNs). Tissues were cultured on MacConkey agar, colistin nalidixic acid agar supplemented with 5% sheep red blood cells and Sabouraud's dextrose agar supplemented with gentamicin. Microbes were identified by standard techniques (Winn *et al.*, 2006); rose-coloured colonies on CHROMagar Candida and lack of filamentation (standard tests for filamentation) confirmed that the recovered yeast was *C. glabrata*.

Mouse models of caecal colonization. To facilitate intestinal colonization with *C. glabrata*, mice were given bacitracin, streptomycin and gentamicin in their drinking water for 3 days prior to oral inoculation with 10^7 *C. glabrata* cells (Wiesner *et al.*, 2001; Bendel *et al.*, 2003). This antibiotic regimen typically eliminates all detectable caecal bacteria. Unless otherwise stated, caeca and draining MLNs were cultured 3 days later and antibiotics were continued for the duration of the experiment. To induce extraintestinal dissemination, several models of trauma and/or immunosuppression were superimposed on this model of caecal colonization.

In one experiment, antibiotic-treated mice were randomized into hypoxia or normoxia groups. Immediately following oral inoculation of *C. glabrata*, mice in the hypoxia group were placed for 1 h in a chamber continuously flushed with 10% oxygen, 10% carbon dioxide and 80% nitrogen; mice were also placed in this environment for 1 h twice daily for the next 2 days and then sacrificed the following day. Control mice were placed in the chamber for the same time periods in room air. In another experiment, mice colonized with *C. glabrata* were injected intraperitoneally (i.p.) 16 h before sacrifice with 100 µg *Escherichia coli* O111:B4 LPS (List Biological Laboratories) suspended in 0.5 ml sterile saline; control mice received sterile saline. In a subsequent experiment, LPS was combined with either hypoxia (described above) or surgical trauma. To induce surgical trauma, mice were anaesthetized with 500 µg xylazine and

2.5 mg ketamine. The caecum and terminal ileum were exteriorized, gently manipulated for 1 min and the intestines returned to the peritoneal cavity. The incision was closed with clips and the mice were sacrificed the following day.

To assess the effect of immunosuppression, one group of mice was injected i.p. (at the time of oral inoculation with *C. glabrata*) with a monoclonal anti-granulocyte antibody, namely 150 µg rat-anti-mouse Ly-6G (Gr-1) and Ly-6C (PharMingen) in 0.5 ml Hanks' balanced salts solution; control mice received 150 µg BSA. In the same experiment, another group of mice was injected i.p. with 0.5 ml containing 2 mg (100 mg kg^{-1}) dexamethasone sodium phosphate (Elkins-Sinn) twice daily for 2 days before sacrifice. (According to the product insert, the LD₅₀ is 800 mg kg^{-1} in adult female mice.) In a subsequent experiment, mice were injected with dexamethasone for a longer period (4 days), and half of these mice also received 100 µg LPS 16 h before sacrifice, whilst the other half received sterile saline. In the same experiment, another group of mice was injected i.p. with 0.5 ml containing 5 mg (250 mg kg^{-1}) cyclophosphamide monohydrate (Sigma-Aldrich) 5 days before sacrifice (maximal bone marrow suppression; Mazur *et al.*, 2002) and these mice also received 100 µg LPS 16 h before sacrifice.

To assess extraintestinal dissemination of *C. glabrata* in mice with active bacterial dissemination, mice were divided into four groups: untreated mice orally inoculated with *C. glabrata*, mice pre-treated with oral antibiotics before oral inoculation with *C. glabrata*, mice pre-treated with oral antibiotics (bacitracin and streptomycin) before oral inoculation with 10^9 streptomycin-resistant *Escherichia coli* M21 cells and mice pre-treated with oral antibiotics before oral inoculation with both *C. glabrata* and *Escherichia coli*. Mice were sacrificed 2 days later, as previous studies have shown that the majority of mice have translocating *Escherichia coli* at this time point in this model (Wells *et al.*, 1987).

Statistical analysis. Fractional data were analysed by χ^2 analysis with continuity correction. Microbial numbers were converted to log₁₀ and analysed by an unpaired Mann–Whitney (two groups) or Kruskal–Wallis test followed by unpaired Mann–Whitney post hoc (more than two groups). Statistical significance was $P < 0.05$.

RESULTS AND DISCUSSION

Intravenous inoculation of *C. glabrata* in mice

To assess systemic virulence of the *C. glabrata* strain used in this study, mice were injected intravenously with 5×10^7 *C. glabrata*. This yeast persisted in the kidney and liver with no significant decrease in fungal burden throughout the 15 day experiment. *C. glabrata* was recovered from the MLN complex, although the numbers declined over time (Table 1). Brieland *et al.* (2001) reported similar results with a clinical isolate of *C. glabrata*, i.e. persistence without significant net growth in a variety of mouse tissues for 21 days after intravenous inoculation. Thus the *C. glabrata* strain used in these studies had a systemic virulence similar to that of a clinical *C. glabrata* isolate.

Oral inoculation of *C. glabrata* in antibiotic-treated mice

In an initial experiment, antibiotic-treated mice were orally inoculated with *C. glabrata* and then placed under

Table 1. Number of *C. glabrata* in the tissues of mice sacrificed 1, 8 and 15 days after intravenous inoculation of 5×10^7 *C. glabrata*

Days after inoculation	No. of mice	No. of <i>C. glabrata</i> in tissue (% positive mice)*		
		Kidney†	Liver†	MLN‡
1	10	4.2 ± 0.6 (90)	5.0 ± 0.7 (90)	1576 ± 661 (60)
8	10	4.7 ± 0.6 (70)	4.2 ± 0.5 (90)	792 ± 703 (60)
15	9§	3.9 ± 0.4 (60)	3.3 ± 0.3 (60)	20 ± 4 (44)

*Lower limits were 2.5 and 2.1 $\log_{10}$ *C. glabrata* cells (g kidney) $^{-1}$ and (g liver) $^{-1}$, respectively, and 10 *C. glabrata* cells per MLN. Parentheses indicate the percentage of mice in which *C. glabrata* was recovered from tissue.

†Mean $\pm$ SEM $\log_{10}$ (g tissue) $^{-1}$.

‡Mean $\pm$ SEM per MLN.

§One death on day 3.

||Significantly decreased compared with day 1 ($P < 0.01$).

normoxic (room air) or hypoxic conditions. Hypoxia was used because mesenteric ischaemia compromises intestinal barrier function, which in turn has been reported to facilitate extraintestinal microbial dissemination (Magnotti & Deitch, 2005). In both treatment groups, *C. glabrata* colonized the caecum at approximately 10^7 cells (g caecum) $^{-1}$ (Table 2, Experiment 1). This concentration was probably close to the maximum obtainable, as, following oral inoculation with *C. albicans*, germ-free mice (with no competing microflora) become colonized with 10^7 *C. albicans* cells (g caecum) $^{-1}$ (Helstrom & Balish, 1979). (In all experiments in Table 2, *C. glabrata* was

the only microbe recovered from mouse caeca.) In mice kept in room air, dissemination of *C. glabrata* to the draining MLNs was not detected and hypoxia had no significant effect on dissemination (Table 2, Experiment 1). As culture of MLNs is considered to be one of the more sensitive indicators of intestinal dissemination and as *C. glabrata* survived in the MLNs of intravenously injected mice (Table 1), failure to recover yeast from MLNs indicated an absence of extraintestinal dissemination. In a previous study using this model, similar hypoxic treatment facilitated extraintestinal dissemination of a mutant strain of *C. albicans* that does not readily form filaments and, like

Table 2. Effect of immunosuppression and/or trauma on caecal colonization and dissemination of *C. glabrata* to the MLNs of mice with antibiotic-induced caecal overgrowth

Experiment	Treatment	Mean $\pm$ SEM $\log_{10}$ <i>C. glabrata</i> cells (g caecum) $^{-1}$ (n)	No. of mice with <i>C. glabrata</i> in MLNs/total no. of mice* (%)
1	Normoxia	6.9 ± 0.1 (8)	0/24 (0)
	Hypoxia	6.8 ± 0.3 (8)	1/24 (4)
2	i.p. saline	6.6 ± 0.2 (8)	0/24 (0)
	i.p. LPS	8.1 ± 0.1 † (8)	2/24 (8)
3	i.p. LPS + surgery	8.0 ± 0.3 (8)	4/23‡ (17)
	i.p. LPS + hypoxia	7.6 ± 0.2 (4)	0/12 (0)
4	i.p. BSA	7.0 ± 0.3 (4)	0/10 (0)
	i.p. anti-granulocyte serum	7.1 ± 0.3 (4)	0/10 (0)
	i.p. dexamethasone (2 days)	7.6 ± 0.2 (4)	0/10 (0)
5	i.p. cyclophosphamide + i.p. LPS	7.8 ± 0.1 (4)	0/12 (0)
	Dexamethasone (4 days)	7.8 ± 0.1 (4)	7/12§ (58)
	i.p. dexamethasone (4 days) + i.p. LPS	8.1 ± 0.1 (4)	7/10§ (70)

*Number of *C. glabrata* recovered from MLNs varied from 10 to 4800 per MLN.

†Significantly increased compared with mice injected with saline ($P < 0.01$).

‡One death in 24 mice.

§Significantly increased compared with mice treated with cyclophosphamide plus LPS ($P < 0.01$).

||Two deaths in 12 mice.

C. glabrata, exists primarily in the yeast form (Kim *et al.*, 2003). Thus we were surprised that hypoxia had no significant effect on dissemination of *C. glabrata* yeast cells.

Parenteral injection of rodents with LPS is often used to mimic septic shock and this treatment results in extra-intestinal dissemination of normal enteric bacteria, such as *Escherichia coli* (Deitch *et al.*, 1991). Antibiotic-treated mice colonized with caecal *C. glabrata* were given parenteral LPS or saline. Saline-treated mice appeared normal, whilst LPS-treated mice had characteristic lethargy, diarrhoea and ruffled fur, confirming the biological activity of LPS. Compared with saline-treated mice, LPS-treated mice had a nearly 32-fold increase in the number of caecal *C. glabrata* cells, but dissemination to MLNs was not significantly increased (Table 2, Experiment 2). Similar results were obtained using a clinical isolate of *C. glabrata* (data not shown). This LPS-induced increase in the number of caecal *C. glabrata* was in contrast to a previous study using a similar mouse model where LPS did not increase caecal colonization of a wild-type strain of *C. albicans* that readily formed filaments in caecal contents; however, LPS was associated with increased caecal concentrations of a mutant *C. albicans* that was defective in filament formation and was seen in caecal contents solely in the yeast form (Henry-Stanley *et al.*, 2003).

As systemic infection with *C. glabrata* is prevalent in post-surgical patients and surgical trauma (laparotomy) facilitates dissemination of enteric bacteria in rodents (Salman *et al.*, 1992), antibiotic-treated mice colonized with caecal *C. glabrata* were subjected to laparotomy. As preliminary experiments indicated that simple laparotomy did not facilitate dissemination of caecal *C. glabrata* (data not shown), this surgical insult was combined with parenteral LPS. Dissemination of *C. glabrata* was not significantly increased in mice given LPS coupled with a surgical insult (Table 2, Experiment 3). In a companion treatment group, mice treated with parenteral LPS plus hypoxia also did not have noticeable dissemination of *C. glabrata* (Table 2, Experiment 3). These two double-hit models are clinically relevant because patients with systemic *C. glabrata* are often severely debilitated from multiple clinical insults (Fidel *et al.*, 1999; Blot *et al.*, 2001). In addition, there is substantial evidence that the biological responses of rodents to two-hit models (e.g. trauma plus sepsis) differ dramatically from responses to a single insult (Mishima *et al.*, 1997; Dienstknecht *et al.*, 2004; Suzuki *et al.*, 2006). It was thus surprising that double-hit models involving LPS plus another insult (surgical trauma or hypoxia) did not induce dissemination of caecal *C. glabrata*.

The Gr-1 antigen is a specific marker for granulocytes (neutrophils, eosinophils and immature monocytes). Mice were injected with Gr-1 antiserum at a dose reported to cause profound depletion of blood and splenic granulocytes in mice for up to 5 days (Czuprynski *et al.*, 1994), whilst another group of mice was given 100 mg dexamethasone kg⁻¹ twice daily for 2 days before sacrifice.

Neither treatment facilitated dissemination of caecal *C. glabrata* (Table 2, Experiment 4).

Although cyclophosphamide has been reported to augment extraintestinal dissemination of *Enterococcus faecalis* in mice with antibiotic-induced *Enterococcus faecalis* overgrowth (Miyazaki *et al.*, 2001), dissemination of *C. glabrata* was not detected in mice given a similar dose of cyclophosphamide along with LPS i.p. (Table 2, Experiment 5). We reported previously that 4 days of dexamethasone treatment twice daily increased extraintestinal dissemination of *C. albicans* in mice with antibiotic-induced caecal overgrowth (Bendel *et al.*, 2002, 2003) and a similar result was noted with *C. glabrata*, although additional treatment with LPS did not have a significant additive effect (Table 2, Experiment 5). Thus extraintestinal dissemination of *C. glabrata* was facilitated by 4 days (but not 2 days) of high-dose dexamethasone treatment, but was not facilitated by cyclophosphamide or anti-granulocyte serum.

As caecal *C. glabrata* did not disseminate in the majority of the above clinically relevant models, we tested the ability of this yeast to disseminate in mice by active dissemination of caecal *Escherichia coli*. In antibiotic-treated mice orally inoculated with both *Escherichia coli* and *C. glabrata*, caecal *Escherichia coli* replicated to high numbers [10^9 – 10^{10} (g caecum⁻¹)] and the incidence of dissemination to the MLNs was high (Table 3). Although the high numbers of caecal *Escherichia coli* seemed to limit the numbers of caecal *C. glabrata*, this yeast was not recovered from the MLNs of mice with active dissemination of *Escherichia coli*.

Thus in several models of trauma and immunosuppression representing a variety of host defence alterations, *C. glabrata* did not often disseminate from the intestinal tract of mice harbouring *C. glabrata* as the only detectable caecal microbe. One exception was mice treated with high doses of dexamethasone, where *C. glabrata* was recovered from the MLNs of the majority of the mice. The reason for caecal *C. glabrata* dissemination following one form of immunosuppression but not another was beyond the scope of the present study, but experiments designed to clarify this issue might yield important information regarding the pathogenesis of *C. glabrata*.

There are several caveats to our general experimental design. First, mice were typically sacrificed 3 days after oral inoculation with *C. glabrata* and it is possible that longer time periods might yield different results. Secondly, systemic *C. glabrata* infections are often associated with old age and chronic disease (Weinberger *et al.*, 2005) and our models used young adult mice given acute insults. Thus additional investigations are needed before these data can be extrapolated to humans. However, although the intestinal tract has been considered a primary portal of entry for systemic infection with *Candida* species, this paradigm may not apply to *C. glabrata* as frequently as it applies to other *Candida* species, including *C. albicans*. It

Table 3. Caecal colonization and extraintestinal dissemination of *Escherichia coli* and *C. glabrata* in antibiotic-treated mice

Oral antibiotics and/or oral microbial inoculum	Mean $\pm$ SEM log ₁₀ microbes (g caecum) ⁻¹ (n=5 per group)			Percentage of mice with microbes in MLN (n=10 per group)	
	Gram-negative bacteria	Gram-positive bacteria	<i>C. glabrata</i>	<i>E. coli</i> *	<i>C. glabrata</i>
<i>C. glabrata</i> only	5.4 $\pm$ 0.3	7.8 $\pm$ 0.1	ND	0	0
Antibiotics plus <i>C. glabrata</i>	ND	ND	7.6 $\pm$ 0.3	0	0
Antibiotics plus <i>E. coli</i>	9.2 $\pm$ 0.1†	3.6 $\pm$ 0.1‡	ND	80§	0
Antibiotics plus <i>E. coli</i> and <i>C. glabrata</i>	9.0 $\pm$ 0.1†‡	3.9 $\pm$ 0.5‡	3.9 $\pm$ 0.4	60§	0

ND, None detected.

*Between 10 and 70 *Escherichia coli* per positive MLN.

†Only streptomycin-resistant *Escherichia coli* detected.

‡Only spore-forming *Bacillus* spp. detected.

§Significantly increased compared with mice given *C. glabrata* alone (with and without antibiotics) ($P \leq 0.01$).

||Significantly decreased compared with mice given antibiotics plus *C. glabrata* alone ($P < 0.01$).

would be challenging to design a study to confirm the clinical relevance of these data obtained from laboratory mice, but the results may be important. If the intestinal tract is not often a primary portal of entry for systemic infection with *C. glabrata*, more attention should be directed to defining the role of *C. glabrata* in sites such as the oral cavity, vagina, skin and indwelling catheters.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service grant GM 059221 from the National Institutes of Health and by the March of Dimes Birth Defects Foundation grant #6-FY04-53.

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