Susceptibility of gnotobiotic transgenic mice (Tgε26) with combined deficiencies in natural killer cells and T cells to wild-type and hyphal signalling-defective mutants of Candida albicans

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Germfree transgenic epsilon 26 mice (Tgε26), deficient in natural killer cells and T cells, were colonized (alimentary tract) with Candida albicans wild-type or each of two hyphal transcription factor signalling mutant strains (efg1/efg1, efg1/efg1 cph1/cph1). Each Candida strain colonized the alimentary tract, infected keratinized gastric tissues to a similar extent, and induced a granulocyte-dominated inflammatory response in infected tissues. Both wild-type and mutant strains formed hyphae in vivo and were able to elicit an increase in cytokine [tumour necrosis factor alpha, interleukin (IL)-10 and IL-12] and chemokine (KC and macrophage inflammatory protein-2) mRNAs in infected tissues; however, administration of the wild-type strain was lethal for the Tgε26 mice, whereas the mice colonized with the mutant strains survived. Death of the Tgε26-colonized mice appeared to be due to occlusive oesophageal candidiasis, and not to disseminated candidiasis of endogenous origin. In contrast, the mutant strains exhibited a significantly reduced capacity to infect (frequency and severity) oro-oesophageal (tongue and oesophagus) tissues. Therefore, the two hyphal signalling-defective mutants were less able to infect oro-oesophageal tissues and were non-lethal, but retained their ability to colonize the alimentary tract with yeast and hyphae, infect keratinized gastric tissues, and evoke an inflammatory response in orogastric tissues.

INTRODUCTION

Mucosal and systemic candidiasis is a serious clinical problem for an increasing number of patients with a variety of congenital, acquired or iatrogenic immunodeficiencies (Wenzel, 1995). Candida species rank fourth among the microbes isolated from blood cultures (Edmond et al., 1999), and systemic infections are associated with an extremely high crude and attributable mortality rate. Although oral antibiotic therapy, age and a variety of immunodeficiencies increase a patient’s risk for candidiasis (Pfaller, 1995; Sanglard, 2002; Wenzel, 1995), very little is known about how and why these factors predispose patients to a range of Candida infections (mucosal, systemic and cutaneous). The innate and acquired immune mechanisms that confine Candida as a commensal to the alimentary tract, and the virulence mechanisms that play a role in the transition from commensal to lethal mucosal and systemic pathogen, are still poorly understood.

Animal studies of candidiasis using genetically engineered Candida albicans strains have been pivotal in defining the virulence traits and morphogenetic characteristics deemed necessary for infection. For example, the hyphal signalling-defective mutant strains efg1/efg1 and efg1/efg1 cph1/cph1, which have a reduced capacity to form hyphae in vitro (Chen et al., 2000; Lo et al., 1997; Riggle et al., 1999), are defective in the production of specific secreted virulence factors (Felk et al., 2002; Staib et al., 2002), have a reduced capacity to adhere to and invade tissues (Dieterich et al., 2002), and exhibit altered virulence in animal models of candidiasis (Bendel et al., 2003; Lo et al., 1997; Riggle et al., 1999). Moreover, the construction of

Abbreviations: GF, germfree; IL, interleukin; MIP, macrophage inflammatory protein; NK, natural killer; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TNFα, tumour necrosis factor alpha.
strains 'locked' into the yeast form (Lo et al., 1997), the filamentous form (Braun et al., 2000) and the generation of regulatable morphogenetic forms, has suggested that it is not the individual morphology per se that is the primary factor required for disease, but rather the yeast to hypha morphogenetic switch (Saville et al., 2003). However, most animal studies of candidiasis determine virulence by parental models of infection (Braun et al., 2000; Felk et al., 2002; Lo et al., 1997; Saville et al., 2003), and therefore bypass the alimentary tract, in which the vast majority of life-threatening Candida infections originate (Marco et al., 1999; Voss et al., 1994). This is because conventional animal models of alimentary tract candidiasis are difficult to work with due to the requirement for broad-spectrum antibiotics (to suppress the Candida-inhibitory microbial flora) and immunosuppressive drugs. The latter agents are known to ulcerate epithelial tissues and cause undefined effects in innate and acquired defence mechanisms. In contrast, germfree (GF) mice do not require antibiotics or immunosuppressive agents. The GF mice have no Candida-inhibitory microbial flora, and mice with defined, innate and/or acquired immune system defects are available, and have been shown to be susceptible to mucosal and systemic candidiasis of endogenous origin (Balish et al., 2001, 2005; Cantorna et al., 1990; Cantorna & Balish, 1990).

In this study of mucosal candidiasis, we assessed the pathogenicity of two wild-type and two hyphal signalling-defective mutant (efg1/efg1, efg1/efg1 cph1/cph1) strains of C. albicans in a gnotobiotic mouse model of candidiasis, which has defects in natural killer (NK)-cell and T-cell production. The pathogenicity of the Candida strains, as determined by alimentary tract colonization, tissue tropism, tissue infectivity, invasiveness, lethality and inflammatory response at sites of infection, is described herein.

RESULTS AND DISCUSSION

Alimentary tract colonization

GF Tg26 mice were quickly (positive faecal samples at 24 h after oral inoculation) and chronically (up to 77 days) colonized by each of the C. albicans strains. Alimentary tract contents (caecum and stomach) had similar viable counts (c.f.u.) for mice infected with either the wild-type (CAF2-1) or each of the mutant strains (Fig. 1). Yeasts and hyphae were detected (Gram stain) in all intestinal-tract specimens assayed at weekly intervals, and when mice were euthanized.

Lethality

CAF2-1 was significantly (P<0.05; Kaplan–Meier test) more lethal for the Tg26 mice than the efg1/efg1 and efg1/efg1 cph1/cph1 mutant strains (Fig. 2). CAF2-1-colonized mice were moribund within 2–3 weeks after oral colonization. Lethality was not due to progressive disseminated candidiasis of endogenous origin, since no viable C. albicans was cultured from homogenates of the internal organs (kidney, liver, spleen or lung) of moribund mice. Death of CAF2-1-colonized mice was apparently due to culturing serial dilutions of the faecal pellets onto SDA showed that the GF mice became colonized (alimentary tract) within 24 h after inoculation. The number of C. albicans c.f.u. in the stomach (after the contents had been removed) and caecum was measured after disruption of the tissues in PBS using a Stomacher II (Fisher Scientific). Internal organs (liver, kidney and spleen) were also homogenized to check for systemic candidiasis of endogenous origin. The homogenates were serially diluted and plated on SDA. The number of c.f.u. was counted after incubation at 37 °C for 24–48 h, and is presented as the log10 viable C. albicans c.f.u. (g dry weight tissue or contents) −1.

Histopathology. Tissue samples (tongue, palate, oesophagus, stomach, liver, kidney, spleen and lung) were collected aseptically and fixed in 10 % formaldehyde in PBS. The tissues were processed in graded (100, 95, 80 and 70 %) alcohol and xylene solutions, and embedded in paraffin. Tissue sections (5 μm) were stained using a standard periodic acid–Schiff reaction (for fungi) counterstained with haematoxylin, and three sections were scored for each tissue. The level of Candida infection (yeast and hyphae) per high powered field (×400) was ranked by a clinical pathologist, as follows: 0, no Candida detected; 1, 1–10 Candida detected; 2, 10–50 Candida detected; 3, 50–100 Candida detected; and 4, confluent Candida detected.

Gene expression by RT-PCR. RNA preparation, RT-PCR competitive methodology and the corresponding primers have been described previously (Balish et al., 2005; Schofield et al., 2004, 2005). Relative RT-PCR was utilized only when expression in one of the experimental groups was low to undetectable, and was therefore unsuitable for measurement by competitive RT-PCR.

Statistics. Statistical analyses were performed using Sigma Stat version 2.0 (SPSS Science) statistical software. P values were calculated using Student’s t test, and Mann–Whitney rank sum, Fisher’s exact, and Kaplan–Meier tests to assess the statistical significance. P<0.05 was considered significant.

METHODS

Micro-organisms. The isogenic C. albicans strains SC5314 and CAF2-1 were obtained from Dr J. Dolan (Nashville State Community College, Nashville, TN, USA), while the efg1/efg1 (HLC52, URA3 heterozygote) and efg1/efg1 cph1/cph1 (HLC54, URA3 heterozygote) mutant strains were kindly provided by Dr G. Fink (Massachusetts Institute of Technology, Boston, MA, USA). C. albicans SC5314 and CAF2-1 were grown on Sabouraud dextrose agar or broth (SDA or SDB; Difco), whereas the mutants were maintained by monthly transfer on minimal synthetic dropout medium (BD Biosciences).

Mice. All transgenic epsilon 26 (Tg26) mice used in this study were derived into the GF state by Caesarian section, and maintained at the Gnotobiotic Research Laboratory, Medical University of South Carolina. A GF colony of Tg26 mice was established with mice obtained from Dr C. Terhorst (Harvard Medical School, Boston, MA, USA). The Tg26 mice were originally generated by overexpressing the full-length human CD3ε gene in C57BL/6 × CBAJF1 mice (Wang et al., 1994). Tg26 mice are defective in both NK and T cells.

Animal model of oro-oesophageal and gastric candidiasis. GF mice were colonized with a pure culture of C. albicans by oral inoculation with 106 viable cells ml−1 for 3 h in drinking water.
occlusive oesophageal candidiasis. In contrast, mice colonized with either mutant strain survived the duration of the study (up to 77 days), and appeared outwardly healthy with no signs (culture and histology) of dissemination to internal organs.

Mucosal infectivity: oro-oesophageal and gastric candidiasis

Tg26 mice exhibited different susceptibilities to oro-oesophageal (tongue, palate and oesophagus) and gastric (cardiac–antrum section) candidiasis when infected with the wild-type or hyphal signalling-defective mutant strains (Table 1, Fig. 3). None of the Candida strains infected the glandular section of the stomach, small or large intestine, caecum or vagina. Interestingly, no significant (P>0.05; Fisher’s exact test) differences in the gastric infectivity (number of tissues infected) were evident with CAF2-1 or the two mutants (Table 1, Fig. 3); however, the Tg26 mice colonized with the mutant strains had significantly fewer infected oesophagi and tongues. The decreased capacity of the mutants to infect oro-oesophageal tissues was not due to a reduced ability to colonize the murine alimentary tract or an inability to form hyphae, since c.f.u. in the alimentary tract were similar, all three strains produced hyphae, and all three strains infected keratinized gastric tissues (Table 1, Fig. 3).

Histopathology scores: severity of oro-oesophageal and gastric candidiasis

CAF2-1 histopathology scores (number of yeast and hyphae in the infected tissues) reflected more severe, invasive candidiasis in the tongue, oesophagus and keratinized gastric tissues than in infected tissues from the efg1/efg1-, or efg1/efg1 cph1/cph1-colonized mice (Table 2). Both mutants had significantly (P<0.05) less severe oesophageal, tongue and gastric infections compared to CAF2-1. Thus, even in the presence of comparable numbers of c.f.u. in the alimentary tract, obvious differences were evident in the severity of the candidiasis produced in oro-oesophageal and keratinized gastric tissues between the wild-type and mutant strains.

Host response to infection

Other studies have indicated that the host responds differentially to Candida infection depending on the morphogenetic form, and the degree of invasiveness and tissue damage (Schaller et al., 2005; van der Graaf et al., 2005; Villar et al., 2005). Therefore, the host response of Tg26 mice to a lethal infection with the clinical isolate SC5314 or non-lethal infection with the mutant efg1/efg1, or efg1/efg1 cph1/cph1-colonized mice (Table 2). Both mutants had significantly (P<0.05) less severe oesophageal, tongue and gastric infections compared to CAF2-1. Thus, even in the presence of comparable numbers of c.f.u. in the alimentary tract, obvious differences were evident in the severity of the candidiasis produced in oro-oesophageal and keratinized gastric tissues between the wild-type and mutant strains.
found no significant difference in cytokine or chemokine expression at the site of infection when a lethal (SC5314) or non-lethal (efg1/efg1 cph1/cph1) orogastric infection was in progress. Each of the Candida strains was able to evoke a prominent, chronic, granulocyte-dominated, inflammatory response in all infected tissues (Fig. 3) (no abscesses were observed in uninfected, but colonized, glandular stomach tissues and the intestinal tract).

Our studies revealed that each of the two hyphal signalling mutants colonized the alimentary tract, formed hyphae, and infected lingual, oesophageal, palate and keratinized gastric tissues to varying degrees; however, candidiasis was less severe (histopathology score) than that in CAF2-1-infected tissues. None of the Candida strains infected the glandular stomach tissue, small or large intestine, caecum or vagina of the Tg26 mice, and were not able to cause a

### Table 1. Infectivity of C. albicans for oro-oesophageal and gastric tissues in Tg26 mice

<table>
<thead>
<tr>
<th>C. albicans strain</th>
<th>Time*</th>
<th>Infected tissue†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tongue</td>
</tr>
<tr>
<td>None (GF)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>14–21†</td>
<td>14/14 (100)</td>
</tr>
<tr>
<td>efg1/efg1</td>
<td>14–77§</td>
<td>11/24 (46)∥</td>
</tr>
<tr>
<td>efg1/efg1 cph1/cph1</td>
<td>14–70§</td>
<td>9/24 (38)∥</td>
</tr>
</tbody>
</table>

*Days after oral inoculation.
†Number of tissues infected/number of tissues examined (percentage of tissues with candidiasis, both yeast and hyphae).
‡Euthanasia was necessary 2–3 weeks after oral inoculation.
§Mice survived the duration of study (up to 11 weeks), and appeared normal and healthy.
∥Significant decrease (P<0.05) compared to CAF2-1 (Fisher’s exact test).
progressive systemic infection of the internal organs. The inability of *C. albicans* to invade all alimentary tract mucosal tissues demonstrates the lack of a common mucosal immune system for resistance of the alimentary tract tissues to *Candida* species (Fidel & Finkel-Jimenez, 2006). Therefore, the Tg<sup>e26</sup> mice, deficient in NK and T cells, retained resistance (partial) to gastrointestinal, and to systemic candidiasis of endogenous origin after alimentary tract colonization with the *Candida* wild-type or mutant strains.

Our findings in Tg<sup>e26</sup> mice regarding the infectivity of the mutants complement but also differ from other studies (Bendel et al., 2003; Kim et al., 2003; Riggle et al., 1999).

For example, Bendel *et al.* (2003) found that the *efg1/efg1 cph1/cph1* mutant and wild-type strain (CAF2-1) colonized the alimentary tract (caecum) to a similar extent, but the mutant was more invasive (extraintestinal dissemination, as measured by the frequency and number of c.f.u. in the kidney) in an antibiotic/immunosuppressed (dexamethasone) conventional mouse model of candidiasis. In a related study, the same group found that the *efg1/efg1 cph1/cph1* strain did not form hyphae in the alimentary tract of antibiotic-treated mice, suggesting that the environmental signal(s) needed to trigger a yeast to hyphal transition was not present in (or was eliminated from) the gut (Kim *et al.*, 2003). Andrutis *et al.* (2000) and Riggle *et al.* (1999) used immunosuppressed (methylprednisone and cyclosporin)

<table>
<thead>
<tr>
<th><em>C. albicans</em> strain</th>
<th>Time*</th>
<th>Histopathology score†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tongue</td>
<td>Palate</td>
</tr>
<tr>
<td>None (GF)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>14–21§</td>
<td>2.8/0.4 (14)</td>
</tr>
<tr>
<td><em>efg1/efg1</em></td>
<td>14–77§</td>
<td>1.0/0.2 (24)</td>
</tr>
<tr>
<td><em>efg1/efg1 cph1/cph1</em></td>
<td>14–70§</td>
<td>0.9/0.3 (24)</td>
</tr>
</tbody>
</table>

*Days after oral inoculation.
†Mean histopathology score/se (number of mice).
§Euthanasia was necessary 2–3 weeks after oral inoculation.
§Mice survived the duration of the study (up to 11 weeks), and appeared normal and healthy.
||Significant decrease (*P*<0.05) compared to CAF2-1 (Student’s *t* test and Mann–Whitney rank sum test).

Fig. 4. Cytokine and chemokine gene expression in GF and *Candida*-infected tissues harvested from Tg<sup>e26</sup> mice 4 weeks after oral association with SC5314 or *efg1/efg1 cph1/cph1*. (a) Competitive RT-PCR analysis was used to compare TNF<sub>x</sub>, IL-10, IL-12, KC and MIP-2 expression in gastric tissues. Numbers represent the mean of three individual stomachs ± SE. *Indicates *P*<0.05 (Student’s *t* test) for tissues from *Candida*-infected mice compared to tissues from GF control mice. Grey bars, GF; white bars, SC5314; black bars, *efg1/efg1 cph1/cph1*. (b) Relative RT-PCR analysis of chemokine (KC and MIP-2) and cytokine (TNF<sub>x</sub>) gene expression in oral (tongue) tissues. The analysis was performed using three tongues from three mice for each group. A PCR reaction was also performed in the absence of cDNA template (1). IL-10 and IL-12 gene expression levels did not increase in tongue tissues (*n* = 2) infected with SC5314 or *efg1/efg1 cph1/cph1* strains compared to GF control tissues (data not shown). M, 100 bp DNA ladder.
gnotobiotic piglets and found similar levels of alimentary colonization with the mutant (efg1/efg1 cph1/cph1) and control (SC5314) strains, and similar to our data, they detected reduced virulence with the mutant strain; however, in contrast to our study, intestinal tract lesions were observed and the gnotobiotic immunosuppressed piglets succumbed to systemic candidiasis. Therefore, important virulence, morphological and pathogenic differences are evident when gnotobiotic and immunosuppressed piglets, antibiotic-treated and immunosuppressed mice or genetically engineered gnotobiotic Tg26 models are used to study the pathogenesis of C. albicans for oro-oesophageal, gastric and systemic tissues after alimentary tract colonization.

The significantly decreased severity of mucosal infections by the two hyphal signalling mutants likely allowed the colonized Tg26 mice to survive the 77 day study. The reasons why the two mutants were able to infect gastric tissues as well as, but with less severity than, CAF2-1, and why they were less able to infect oro-oesophageal tissues with yeasts and hyphae is unclear; however, the Efg1 and Cph1 transcriptional regulators are required for the expression of the secreted aspartic proteinase genes SAP4–6, adhesion and invasion of tissues (Dieterich et al., 2002; Felk et al., 2002; Staib et al., 2002). In particular, SAP6 expression, while undetected and presumably unimportant during the early stages of gastric candidiasis, is readily detected during oro-oesophageal candidiasis (Schofield et al., 2003). Therefore, the reduced capacity to invade oro-oesophageal but not gastric tissues could be due to defects in Sap6 virulence factor production. In addition, the results presented here suggest that the ability to readily form hyphae, in addition to producing Efg1/ Cph1-associated virulence factors, may be more important for Candida to successfully infect oro-oesophageal, but not gastric tissues.

A chronic, granulocyte-dominated, inflammatory response was evoked by each of the three Candida strains in infected oro-oesophageal and gastric (keratinized) tissues. The lack of inflammation in uninfected tissues (i.e. glandular stomach, small and large intestine, caecum and vagina) from colonized mice suggests that tissue invasion, as opposed to alimentary tract colonization, with hyphae and yeasts, is necessary to evoke and sustain a chronic granulocytic inflammatory response. The chronic inflammation observed in the infected tissues suggests that chemoattractants were still present on, or were produced by, all of the Candida strains tested. The evoked inflammation was also apparently independent of functional NK or T cells in the host. Both wild-type and mutants strains were also able to induce a cytokine and chemokine response in infected gastric and oral tissues to a similar degree. Other studies have shown that the ability to evoke a proinflammatory response is correlated with the degree of invasion or tissue damage. For example, Villar et al. (2005), using an invasive-deficient (rim101 knockout) or highly invasive (rim101-complemented) C. albicans strain, have demonstrated that highly invasive strains trigger higher levels of proinflammatory cytokine production in oral epithelial cells in vitro than do invasive-deficient strains. Similarly, Schaller et al. (2005), using in vitro reconstituted human vaginal epithelium, have demonstrated that mutants lacking SAP1 or SAP2 exhibit reduced tissue damage and potential to stimulate cytokine expression, while mutants lacking SAP4–6, which are not deficient in their ability to cause tissue damage, stimulate cytokine expression to a similar degree as the wild-type strain. In our study with defined T- and NK-cell-deficient gnotobiotic mice, neither the severity nor the outcome of the infection (lethal or non-lethal) appeared to correlate with the capacity of Candida strains lacking Efg1 or Cph1 to evoke a proinflammatory and chemoattractive response in infected gastric or tongue tissues.

Recently, candidiasis studies have been carried out in GF gp91phox−/−/NOS2−/− mice with combined defects in the production of oxygen and nitrogen metabolites [reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI)], but with intact NK and T cell functions (Balish et al., 2005). The mice with combined ROI and RNI deficiencies succumbed to candidiasis within 2 weeks after their alimentary tracts were colonized with wild-type, efg1/efg1 or efg1/efg1 cph1/cph1 mutant strains. Lethality appeared to be due to a tissue-destructive immune response in their Peyer’s patches, mesenteric lymph nodes and internal organs that was triggered by the translocation of Candida (wild-type and each of the mutant strains) from the intestinal tract into the internal organs. The route of translocation appeared to be via Peyer’s patches and the mesenteric lymph nodes. This latter study (Balish et al., 2005) strongly suggests that phagocytic cells, defective in the ability to produce both ROI and RNI, predispose mice to systemic candidiasis of endogenous origin. Conversely, combined defects in NK and T cells predispose mice to oro-oesophageal and gastric candidiasis, but not to systemic candidiasis of endogenous origin.

Much remains to be learned about host–Candida interactions at mucosal surfaces. The availability of many virulence-defective mutants of Candida and genetically engineered animal models with specific immune defects allows for new and innovative studies on the pathogenesis of Candida, and provides the opportunity for investigation of the prophylaxis and immunotherapy of candidiasis. Such data will contribute to our knowledge about the relative importance of both NK and T cells in murine resistance to oro-oesophageal, gastric and systemic candidiasis of endogenous origin.

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