A Murine Model of \textit{Candida glabrata} Vaginitis

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Vaginal \textit{Candida glabrata} infections have increased significantly in recent years and are particularly common in women with uncontrolled diabetes mellitus. Efforts to understand the pathogenesis and treatment of this infection have been hindered by the lack of experimental animal models. Before onset of hyperglycemia, nonobese diabetic (NOD) mice inoculated intravaginally with clinical \textit{C. glabrata} isolates were shown to support high vaginal titers of \textit{C. glabrata} for $>14$ days with evidence for superficial invasion of vaginal epithelial tissue. In contrast, congenic diabetic-resistant and mice susceptible to \textit{Candida albicans} infections were significantly less susceptible to vaginal infection by \textit{C. glabrata}, suggesting a potential link between the susceptibility of NOD mice to diabetes and their susceptibility to vaginal \textit{C. glabrata} infections. This animal model of \textit{C. glabrata} vaginitis provides a means to study the genetics and pathogenesis of \textit{C. glabrata} infections and to evaluate the efficacy of antifungal agents against \textit{C. glabrata}.

The incidence of mucosal \textit{Candida glabrata} infections has increased in immunocompromised hospitalized patients, including human immunodeficiency virus–infected persons treated prophylactically with fluconazole [1–4]. Vaginal infections caused by \textit{C. glabrata} occur second only to those caused by \textit{Candida albicans} and are the most frequent non–\textit{C. albicans} cause of acute and chronic vulvovaginitis [5–10]. Interestingly, in an analysis of patients seen in the Wayne State University Referral Vaginitis Clinic from 1986 through 1994, the incidence of individual cases of non–\textit{C. albicans} vaginitis ($>90\%$ \textit{C. glabrata}) in human immunodeficiency virus–negative women rose abruptly in 1994 to $29\%$ (unpublished data) after remaining at $\sim 13\%$ from 1986 through 1993 [11].

Persons with diabetes mellitus are particularly susceptible to fungal infections [12, 13], including mucosal and systemic infections by \textit{C. albicans} [14, 15]. Furthermore, uncontrolled diabetes mellitus is a common underlying factor in women with acute, recurrent, and chronic vulvovaginitis caused by both \textit{C. albicans} and \textit{C. glabrata} [5], indicating that diabetes mellitus may also be an important risk factor for \textit{C. glabrata} infections.

Symptomatic \textit{C. glabrata} infections are often difficult to treat, and isolates are frequently less susceptible to antifungal agents in vitro [5, 16]. Thus, efforts have been made to devise and test new antifungal therapeutic strategies. However, progress has been hampered by the lack of an established, reproducible animal model of mucosal \textit{C. glabrata} infections. The purpose of the present study was to establish an experimental murine model of \textit{C. glabrata} vaginitis by evaluating \textit{C. glabrata} vaginal burden and histopathology after inoculation in estrogen-treated mice with intermediate and high susceptibilities to \textit{C. albicans} infection [17–21] and in congenic diabetes-sensitive and -resistant strains of mice [22, 23].

Materials and Methods

\textit{Mice}. Female CBA/J (H-2\textsuperscript{b}) and DBA/2 (H-2\textsuperscript{d}) mice with intermediate and high susceptibilities to \textit{C. albicans} infection, respectively [20, 21], nonobese diabetic (NOD/Lt) (H-2\textsuperscript{g7}) mice [22], and nonobese diabetes-resistant (NOR/Lt) (H-2\textsuperscript{lt}) mice [23], all at 7–8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were used throughout these studies. The NOR strain represents a congenic strain with limited regions of the NOD genome replaced with genes from DBA/2 and C57BL/12.2J mice [23].

\textit{Yeast isolates.} \textit{C. albicans} 3153 A (American Type Culture Collection, Rockville, MD) [17] was used to initiate \textit{C. albicans} vaginitis. Three clinical vaginal isolates of \textit{C. glabrata} (CS 177.93, LF 547.92, and RA279.95) were used to potentiate \textit{C. glabrata} vaginal infections. All isolates had similar patterns of in vitro susceptibility to routinely tested azoles (unpublished data). Two clinical isolates of \textit{Saccharomyces cerevisiae} (RS557.93 and CL256.94) were also used to examine the potential for \textit{S. cerevisiae} vaginal infectivity.

\textit{Vaginal inoculation.} Seventy-two hours before vaginal inoculation, mice (7 weeks old) were treated with the first of weekly subcutaneous injections of 0.4 mg of estradiol valerate (Sigma, St. Louis) in sesame oil. For vaginal inoculations, \textit{C. albicans} (5 $\times$ 10$^5$ cfu), \textit{C. glabrata} (5 $\times$ 10$^5$ or 1 $\times$ 10$^5$ cfu), or \textit{S. cerevisiae} (1 $\times$ 10$^5$ cfu) blastococidia from a stationary-phase culture were administered into the vaginas of CBA/J, DBA/2, NOD/Lt, or NOR/Lt mice in a volume of 20 $\mu$L of PBS as described [17].

\textit{Enumeration of vaginal fungus burden.} Vaginal \textit{C. albicans}, \textit{C. glabrata}, or \textit{S. cerevisiae} burden was quantitated by culture of vaginal lavage fluid [17]. Briefly, each animal was subjected to a vaginal lavage using 100 $\mu$L of PBS with extensive aspiration and gentle scraping of vaginal tissue with the pipette tip. The lavage...
fluid was serially diluted, plated onto Sabouraud dextrose agar, and incubated at 30°C. Colony counts from 48- to 72-h lavage cultures were used to determine vaginal fungus burden. Vaginal lavage fluid from C. albicans-, C. glabrata-, or S. cerevisiae-inoculated mice were also analyzed by blinded microscopy (wet-mount slide preparation) for the presence of cellular infiltration as well as yeast. In addition, slides from C. albicans-inoculated mice were scored for hyphal content: 0, no hyphae; 4+, presence of large hyphal masses. Colonies from lavage cultures were also used to confirm the identity of the respective organisms. C. albicans was identified by a positive germ tube test after a 2-h incubation in 0.5 mL of heat-inactivated fetal calf serum (Life Technologies Gibco BRL, Gaithersburg, MD) at 37°C in 10% CO₂ as described [17]. Germ tube-negative C. glabrata and S. cerevisiae isolates were confirmed biochemically using API 20C yeast assimilation strips (BioMerieux Vitek, Hazelwood, MO).

**Histopathology.** Vaginas from randomized, nonlavaged C. albicans- and C. glabrata-inoculated mice were excised, fixed in 10% formalin, and embedded in paraffin. Paraffin sections were examined serially until high concentrations of yeasts or hyphae were located. Subsequent sections were analyzed by silver staining methods with a commercial kit (Sigma).

**Qualitative assessment of diabetes.** NOD mice were evaluated weekly for urine glucose content using Clinistix test strips (Miles, Elkhart, IN). NOD mice were inoculated 5 weeks before the earliest reported onset of hyperglycemia (12 weeks of age) [22].

**Statistics.** Data were analyzed by the Mann-Whitney U test using a one-tailed test. Significance was defined as \( P < .05 \).

**Results**

**Experimental C. glabrata vaginal burdens in mice.** To determine whether vaginal tissue of various strains of mice could support the presence or growth of C. glabrata, DBA/2 and CBA/J mice with high and intermediate susceptibilities to C. albicans infection, respectively [20, 21], and NOD mice were given a relatively high vaginal inoculum (1 \( \times 10^7 \) cfu) of a clinical C. glabrata vaginal isolate under conditions of pseudoestrus. Vaginal C. glabrata burden was monitored over a 21-day period using 5 mice per group per time period (figure 1). CBA/J mice did not show detectable C. glabrata in any lavage fluid throughout the 21-day period. DBA/2 mice supported small numbers of C. glabrata (10⁵–10⁷ cfu) for a short time (<14 days) with no more than 55% of the animals having positive cultures at any time point. In contrast, NOD mice supported high vaginal titers of C. glabrata (10⁵–10⁷ cfu) for >14 days with a high percentage of animals with positive cultures (90% through day 10 and 70% at day 14). During periods when NOD and DBA/2 mice both had positive vaginal cultures (6 and 10 days after vaginal inoculation), vaginal C. glabrata burden in NOD mice was significantly greater than that in DBA/2 mice (\( P < .037 \)). By day 21, C. glabrata was no longer detectable in lavage fluid of NOD mice. Similar results were observed for NOD mice inoculated with 2 additional clinical isolates of C. glabrata (data not shown). In each case, colonies from quantitative cultures of lavage fluid were germ tube-negative and identified as C. glabrata by API assimilation strips (BioMerieux Vitek).

Additional experiments examined the effect of inoculum (1 \( \times 10^2 \) vs. 5 \( \times 10^3 \) cfu) on the incidence of C. glabrata vaginal burden. Although the high inoculum resulted in increased fungus burden early after inoculation (day 6, \( P < .01 \)), the general kinetic pattern of fungus burden after day 6 was similar with either inocula and any of the 3 C. glabrata isolates (data not shown).

**Differential susceptibility of NOD mice to fungal organisms.** The susceptibility of NOD mice to C. glabrata prompted experiments to examine the ability of NOD mice to support the vaginal presence or growth of other species of fungal organisms with varying levels of pathogenicity. Mice were inoculated intravaginally with C. glabrata (1 \( \times 10^7 \) cfu), S. cerevisiae (1 \( \times 10^7 \) cfu), or C. albicans (5 \( \times 10^5 \) cfu—the inoculum normally given to CBA/J mice). Vaginal fungus burden was monitored for 21 days using 5 mice per group per time period (figure 2). In mice given C. glabrata, the previously described pattern of vaginal fungus burden was evident throughout the 21-day period. In mice given C. albicans, extremely high levels of vaginal C. albicans burden (10⁶–10⁷ cfu) were sustained through the 21-day period, with >85% of the animals having positive cultures at any time, all of which had masses of hyphae (4+ score) in recoverable lavage fluid. Surprisingly, there was a significant level of mortality in NOD mice inoculated with C. albicans; 16% at day 6, 22% at day 10, 4% at day 14, and 11% at day 21. In mice given S. cerevisiae, significantly fewer organisms were recoverable (10⁵ cfu) early after vaginal inoculation and none after day 10. Similar results were observed for a second S. cerevisiae clinical isolate (data not shown). Comparison of fungus burden between estrogen-treated mice

![Figure 1. Experimental C. glabrata vaginal infections in mice with intermediate (CBA/J) and high (DBA/2) susceptibilities to Candida albicans infection and in nonobese diabetic (NOD/Lt) mice. Data points represent mean cfu of animals with positive cultures only (percentage of animals with positive cultures is shown). Data are results of 3 experiments with each strain of mice in parallel, using 5 mice/group/time point. ms, mouse.](image-url)
Figure 2. Comparative analysis of C. glabrata, Candida albicans, and Saccharomyces cerevisiae vaginal fungus burden in nonobese diabetic mice. Data points represent mean cfu of animals with positive cultures only (percentage of animals with positive cultures is shown). Data are cumulative results of 3 experiments with 5 mice/group/time point.

given C. glabrata, S. cerevisiae, or C. albicans revealed significant differences between C. glabrata and C. albicans on day 10 (P < .03) and 21 (P < .001), with fewer C. glabrata–inoculated mice showing positive cultures on days 14 and 20; between C. glabrata and S. cerevisiae at all time periods (P < .002) except day 21; and between C. albicans and S. cerevisiae at all time points (P < .001).

Additional experiments examined the requirement for pseudoestrus in C. albicans– and C. glabrata–inoculated mice. Results showed that on days 6–10 after vaginal inoculation, both estrogen-treated and non–estrogen-treated C. albicans– or C. glabrata–inoculated mice had relatively high vaginal fungus burdens (10^4–10^6 cfu) but that non–estrogen-treated mice had fewer animals showing positive cultures (60% vs. 95%–100%). During later time periods (days 10–21), non–estrogen-treated C. glabrata–inoculated mice became culture-negative sooner than their estrogen-treated counterparts, while non–estrogen-treated C. albicans–inoculated mice retained positive cultures at day 21 in high titer (10^5–10^6 cfu), similar to results with estrogen-treated mice but without the occurrence of mortality and with fewer animals showing positive cultures (20%–40% vs. 86%–100%).

During these same experiments, lavage fluids collected from the respective groups of mice 10 days after vaginal inoculation were examined for cellular infiltration. Results showed that both C. glabrata– and S. cerevisiae–inoculated mice had lymphoid-like cells in the vaginal lavage fluid but at lower levels than that observed in C. albicans–inoculated mice (data not shown).

Histopathology of vaginal tissue from C. glabrata– and C. albicans–inoculated NOD mice. Nonlavaged vaginal tissue from 10-day estrogen-treated C. glabrata– and C. albicans–inoculated NOD mice was examined for the presence of the respective organisms. Estrogen-treated noninoculated mice were used as the negative control group and showed no evidence of yeast (figure 3A). Figure 3B illustrates the presence of blastospores associated with the vaginal mucosa in C. glabrata–inoculated mice; some of these blastospores appeared intraepithelial in vacuole-like vesicles. Vaginal tissue from C. albicans–inoculated mice contained large masses of hyphae superficially associated with the mucosa (figure 3C).

C. glabrata vaginal infection in NOD and insulitis-resistant NOR congenic mice. To examine a putative relationship between the genetic susceptibility of NOD mice to diabetes and their susceptibility to C. glabrata, NOD mice and congenic insulitis-resistant NOR mice were evaluated for the ability to support the vaginal presence of C. glabrata. For this, NOD and NOR mice were given the high vaginal inoculum (1 × 10^7 cfu) of a C. glabrata clinical isolate in the presence of pseudoestrus and monitored for vaginal fungus burden through 14 days (figure 4). NOD mice had significantly higher vaginal levels of C. glabrata than did NOR mice throughout the 14-day period (10^5–10^6 cfu vs. 10^2–10^5 cfu, P < .006). Also, whereas 70%–80% of NOD or NOR mice had positive cultures 6 days after vaginal inoculation, only 17% of NOR mice showed positive vaginal cultures on day 10 compared with 80% of NOD mice. Similarly, on day 14, when NOD mice continued to harbor high numbers of organisms with 50% of the animals infected, NOR mice no longer showed detectable C. glabrata in lavage fluids. Diabetes, as measured by urine glucose levels, was not detected in any NOD mice until 24 weeks of age, representing 14 weeks after vaginal inoculation.

Discussion

The increasing prevalence of non–C. albicans fungal infections in immunocompromised persons [1–4], especially infections caused by C. glabrata, has stressed the importance of establishing reproducible animal models whereby pathogenesis can be studied and therapeutic strategies evaluated. Historically, it has been extremely difficult to establish an animal model of C. glabrata infection [24]; the only successful report is a recent model of disseminated C. glabrata in immunocompromised mice [25]. In the present study, taking into account the susceptibility of persons with uncontrolled diabetes mellitus to acquire vaginal C. glabrata infections, we examined the potential to induce a vaginal C. glabrata infection in NOD mice. Consistent with this premise, NOD mice did in fact support the vaginal presence or growth of multiple C. glabrata isolates significantly better and for longer periods than did other species of mice with high and intermediate susceptibilities to C. albicans.

The property of NOD mice to support the vaginal presence of C. glabrata was extended to include C. albicans and S. cerevisiae. In mice inoculated with C. albicans, a persistent vaginal infection resulted, with >85% of the animals retaining...
Figure 3. Histopathology of vaginal tissue from estrogen-treated nonobese diabetic mice inoculated with *C. glabrata* and *Candida albicans* (silver stain of vaginal tissue sections from 10-day inoculated mice). A, uninoculated; magnification, $\times 40$; B, *C. glabrata*—inoculated; magnification, $\times 100$; C, *C. albicans*—inoculated; magnification, $\times 100$. Arrows, representative blastospores or hyphae.
high numbers of organisms at day 21 and with large masses of hyphae recovered in lavage fluid. In fact, the *C. albicans* burden in lavage fluid from NOD mice was ~10-fold higher than that usually observed in CBA/J mice [17, 18]. Notably, 25% mortality occurred through day 10. This uncharacteristic mortality during a vaginal infection may have been due to the high vaginal titers of *C. albicans* contaminating the bedding, causing potentially lethal *C. albicans* gastrointestinal or other site-specific infections. Alternatively, the high fungus burden and resulting inflammation may have affected bladder or kidney function, as the bladders of infected mice were noticeably enlarged.

The condition of pseudoestrus, while clearly increasing the percentage of animals infected, had little effect if any on the level of fungus burden in *C. glabrata*– or *C. albicans*–infected NOD mice. This is in contrast to the overwhelming effects exogenous estrogen has on *C. albicans* vaginitis in CBA/J mice [18]. Perhaps the high susceptibility of NOD mice to *Candida* species allows the lower endogenous levels of estrogen to more easily support the adherence and growth of *C. glabrata* or *C. albicans*.

It is interesting that NOD mice were capable of supporting the vaginal presence of *S. cerevisiae*, an organism considered by most to be nonpathogenic but nevertheless capable of causing symptomatic vaginitis in rare cases [7, 8]. In fact, the results showed a clear differential pattern of susceptibility to the fungal organisms, greatest to *C. albicans*, intermediate to *C. glabrata*, and lowest to *S. cerevisiae*. These data provide additional evidence that NOD mice are highly susceptible to fungal colonization or infection.

Evidence of vaginal infection versus colonization is notoriously difficult to evaluate in animal models since symptoms cannot be measured. For *C. albicans* infection, since cultured lavage fluid or histopathology of vaginal tissue from normal mice or estrogen-treated mice (figure 3A) are negative for yeast, the persistent presence of *C. albicans* collected from inoculated mice is usually sufficient evidence of infection. Furthermore, because the hyphal form is considered the more pathogenic morphologic form of *C. albicans* [5, 17, 26, 27], the presence of hyphae in lavage fluid serves as additional evidence of infectivity [17]. Histopathology can be used for yet additional evidence of infection, although the fact that hyphae only superficially associate with vaginal tissue [26] limits the information histology can provide.

In animals inoculated with the non–hyphae-producing *C. glabrata*, evidence for infectivity can be potentially more difficult to assess. Nevertheless, the present data in NOD mice favor a state of infection rather than colonization. First, NOD mice had a persistent presence of *C. glabrata* in high titers, while other murine strains given the same inoculum had low numbers of organisms that rapidly became culture-negative or were culture-negative at the first lavage point. Second, the immune response–associated lymphoid-like cellular infiltrations collected in the lavage fluid of *C. glabrata*–inoculated mice was similar to that observed during vaginal infections by *C. albicans*. Third, the histopathologic presence of blastospores associated with the vaginal mucosa, some of which appeared in intraepithelial vacuole-like vesicles, supports the concept that *C. glabrata* had superficially infected the vaginal mucosa.

Diabetic mice have been used to study a number of *Candida* infections, including systemic *C. albicans* and *Candida tropicalis* infections [28–30] and *C. albicans* vaginal infections [31]. In each case, the animals were more susceptible to infection when hyperglycemic. In contrast, vaginal *C. glabrata* infection in NOD mice was established and resolved before the onset of diabetes. In fact, although *C. glabrata* infection in NOD mice cleared by 9 weeks of age, the onset of diabetes did not occur until 24 weeks of age. This apparent lag time to a state of hyperglycemia is not surprising in light of the fact that although the onset of diabetes in NOD mice rarely occurs before 12 weeks of age, trauma and stress (i.e., infection) are known to significantly delay the onset and potentially prevent the occurrence of insulitis [22].

To examine this intriguing susceptibility of NOD mice to *C. glabrata* infections more closely, we did similar analyses in insulitis-resistant NOR mice. NOR mice represent a recombinant congenic strain developed from NOD mice whereby 38% of the genome (8 chromosomes) became contaminated with genes of C57BL/KsJ or DBA/2 origin [32]. As a consequence, the genetic susceptibility to diabetes was lost. Studies are ongoing in NOD and NOR mice to identify the chromosomal regions that confer susceptibility to diabetes.

With the use of these congenic strains of mice, we showed that in contrast to the persistent vaginal *C. glabrata* titers in inoculated NOD mice, inoculated NOR mice had significantly lower fungus burden that became undetectable at day 10. Since similar results were observed in the contaminating strains of NOR mice, DBA/2 (figure 1) and C57BL/6 (unpublished observations), it is intriguing to speculate that the susceptibility of

**Figure 4.** *C. glabrata* fungus burden in nonobese diabetic (NOD) and nonobese insulitis-resistant (NOR) mice. Data are cumulative results of 2 experiments with 4–5 mice/group/time point.
NOD mice to *C. glabrata* vaginal infections before the onset of hyperglycemia is associated with their genetic susceptibility to diabetes. Indirect support for this is garnered by the fact that normal ICR mice made hyperglycemic by streptozotocin were more susceptible to vaginal infections by *C. albicans* [31], and CBA/J similarly treated became susceptible to *C. glabrata* vaginal infection (unpublished data).

Past theories regarding the association of diabetes with susceptibility to fungal infections have focused on clinical manifestations of hyperglycemia and insulin. These included the contribution of glucose, which provides an excellent carbon source for *Candida* growth [33], impaired phagocytic function [13, 34], and T cell deficiencies, taking into account that diabetic patients are known to express abnormal ratios of T cell subsets [35] and the fact that cell-mediated immunity is the predominant host defense mechanism against *Candida* species [36–41]. Thus, to our knowledge, the present data are the first to suggest a potential link between the genetic susceptibility to diabetes mellitus and susceptibility to infections by at least *C. glabrata* and *C. albicans*. If such an association exists clinically, those nondiabetic persons with an inherited potential for acquiring diabetes mellitus might be at increased risk of mucosal infections by *Candida* species.

In summary, this study represents the first reported animal model of *C. glabrata* vaginal infection. NOD mice given a vaginal inoculum of *C. glabrata* before the onset of diabetes developed persistent vaginal *C. glabrata* burdens with good evidence of infectivity over a 14-day period. This model provides a previously unavailable method for studying the in vivo efficacy of oral and topical antifungal agents as well as the immunologic or genetic properties affecting pathogenesis of vaginal *C. glabrata* infections.

References


