

Candida albicans Strain-Dependent Virulence and Rim13p-Mediated Filamentation in Experimental Keratomycosis

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PURPOSE. To compare the virulence of wild-type *Candida albicans* strains in a murine model of corneal candidiasis and to investigate the role of fungal filamentation in disease progression.

METHODS. Scarified corneas of immunocompetent or cyclophosphamide-treated BALB/c mice were topically inoculated with one of three human isolates of *C. albicans*, a homozygous mutant of the pH-dependent filamentation gene *rim13* or a mutant reference strain control. Mock-inoculated eyes served as negative controls. Corneal disease was categorized daily for 8 days with quantitative fungal culturing of eyes at 6 hours, 1 day, 4 days, and 8 days after infection and histopathologic examination at 1 day and 4 days after infection.

RESULTS. Corneal disease severity differed significantly among wild-type strains ($P \leq 0.02$). The *rim13*^{-/-} mutant Tn7-rim13 was fully attenuated, whereas the mutant control DAY286 was fully virulent. Pretreatment of mice with cyclophosphamide increased susceptibility to wild-type *C. albicans* and partially rescued the attenuated phenotype of the genetically deficient *rim13*^{-/-} fungal mutant. All strains replicated with similar kinetics in vitro, and wild-type strains had similar clearance from infected eyes. Histopathologic findings correlated with disease severity.

CONCLUSIONS. Wild-type strains of *C. albicans* that differ significantly in ocular pathogenicity correlate with the ability of yeast to produce pseudohyphae and hyphae and to invade corneal tissue. Full attenuation of the fungal *rim13*^{-/-} mutant is the first direct demonstration of a hyphal morphogenesis-related gene as a specific virulence factor for *C. albicans* during corneal infection. (*Invest Ophthalmol Vis Sci.* 2007;48:774-780) DOI:10.1167/iovs.06-0793

Candida albicans is found on the ocular surface of up to one third of otherwise healthy persons.¹⁻⁴ Although this fungal species is typically commensal, severe ocular disease develops under certain conditions. How this shift to pathogenic invasion

occurs is still unclear, but the transition is finely balanced and genetically regulated.⁵ Fungal mechanisms crucial for mucosal and systemic infection by *C. albicans* include adherence, germination, production of extracellular proteinases and phospholipases, and phenotypic switching from yeasts to pseudohyphae and hyphae.⁶⁻⁹ Previous work has suggested that the conversion to filamentous forms contributes to ocular virulence of *C. albicans*.^{10,11}

Environmental cues such as pH^{12,13} influence filamentation growth of *Candida*, *Saccharomyces*, and *Aspergillus* through a conserved alkaline-response pathway.^{14,15} Mutant strains unable to grow at a given pH in vitro are less capable of infecting selective sites in vivo.^{16,17} This system, called the RIM101 pathway or the PacC pathway, depending on fungal genus,¹⁸ is governed by the zinc finger-containing transcription factor Rim101p/PacC.^{15,19-21} In acidic conditions, Rim101p is full-length and inactive, whereas in neutral to alkaline conditions its C-terminal portion is cleaved, resulting in activation of the protein that regulates gene expression.²²⁻²⁴ Several gene products are required for this proteolytic processing, including Rim8p/PalF, Rim9p/PalI, Rim13p/PalB, Rim20p/PalA, and Rim21p/PalH.^{24,25} Rim13p/PalB is a calpain-like protease that cleaves the C-terminal glutamate-aspartate-rich domain of Rim101p during activation.¹⁸

Because of the complex life cycle of fungi, investigating the pathogenesis of oculomycosis requires in vivo models that allow high reproducibility and sensitive quantitation.^{10,11,26-28} This study compared the degrees of virulence of three different *C. albicans* wild-type strains in mice and evaluated the contribution of the RIM101 signal transduction pathway.^{15,20,21} Our findings provide direct evidence for the importance of filamentation in keratomycosis and demonstrate that a morphogenesis-related fungal gene is a specific virulence factor for *C. albicans* during corneal infection.

MATERIALS AND METHODS

Fungal Cultures

Three wild-type strains of *C. albicans* originally isolated from human infection, a Tn7 transposon mutant, and a mutant control reference strain were evaluated. Strain VE175 is a corneal isolate that has been used to induce experimental keratomycosis in rabbits.²⁹ Strain B311 (ATCC 32354; American Type Culture Collection, Rockville, MD) is an isolate that is highly virulent in mice when injected intravenously³⁰ and that causes ocular disease in mice after corneal inoculation.¹¹ Strain SC5314 has been used extensively for genetic studies of *C. albicans* and experimentally causes corneal disease in rabbits.^{29,31} Strain Tn7-rim13 is a homozygous mutant with a transposon insertion at position 239 of the *rim13* coding sequence¹⁸ that was transformed into strain BWP17,¹⁵ a derivative of strain SC5314, as part of a homozygous insertion mutant library.³² The mutant control reference strain DAY286 has a Ura⁺Arg⁺His⁻ genotype and was created by the transformation of strain BWP17.¹⁵

All yeast strains were grown on Sabouraud dextrose agar (Difco, Detroit, MI) for 3 days at 25°C. For corneal inoculation, the yeasts were harvested and diluted in sterile phosphate-buffered saline (PBS) to yield

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1×10^5 or 1×10^6 colony-forming units (CFU)/5 μ L inoculum based on optical density (OD) at 600 nm and using a conversion factor of 1 U OD₆₀₀ equal to approximately 3×10^7 CFU/mL based on *Saccharomyces*³³ and confirmed for *Candida*.¹¹ For the in vitro growth kinetics study, yeasts were collected from the agar plates and diluted in Sabouraud dextrose broth (Difco).

Animals

Adult female BALB/c mice (Harlan Sprague-Dawley, Houston, TX) were used at 6 to 8 weeks of age. A subset of mice was pretreated 5 days, 3 days, and 1 day before corneal inoculation with intraperitoneal injections of cyclophosphamide (Sigma, St. Louis, MO) at 180 mg/kg body weight, as previously described.¹¹ This regimen and dose of cyclophosphamide results in severe immunosuppression and induces a biological effect within ocular tissues.¹¹ Mice were anesthetized and corneally scarified as previously described.¹¹ Five microliters (1×10^5 or 1×10^6 CFU) inoculum of *C. albicans* was applied to the scarified corneas. Mice that were scarified and received mock inoculation with sterile PBS served as negative controls. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

In Vitro Growth Kinetics

One thousand CFU of each *C. albicans* strain were inoculated into 2 mL Sabouraud dextrose broth and incubated at 27°C with continuous rocking for 7 days. Two milliliters mock-inoculated Sabouraud dextrose broth served as a negative control. Results of two independent experiments performed in triplicate were averaged. The broth was sampled at 6, 9, 22, 34, 50, 76, 96, and 168 hours after inoculation for in vitro growth by measuring the OD₆₀₀ and using the conversion factor of 3×10^7 CFU/mL per 1 U OD₆₀₀.^{11,33} Statistical analyses were determined with Student's *t* test.

Clinical Scoring

All mice were monitored daily for corneal involvement for up to 8 days postinoculation (p.i.). The severity of keratomycosis was scored with the aid of a dissecting microscope. A grade of 0 to 4 was assigned to each of three criteria: area of opacity, density of opacity, and surface regularity.^{11,28} Scores from each category were tallied daily for each eye to yield a possible total score of 0 to 12. A total score of 1 to 5 was categorized as mild eye disease, a total score from 5 to 9 was considered moderate, and a total score greater than 9 was considered severe.¹¹ Results were evaluated for statistical significance by the Kruskal-Wallis one-way analysis of variance on ranks. Pairwise multiple comparison procedures included the Dunn method and the Tukey test.

Quantitative Isolate Recovery

Mice were killed and eyes were enucleated at 6 hours, 1 day, 4 days, and 8 days p.i. and were processed for microbial culturing. Eyes from infected and mock-infected mice were individually ground in a frosted glass grinder, as previously described.¹¹ A 0.5-mL homogenate aliquot was fourfold serially diluted in Sabouraud dextrose broth containing chloramphenicol 50 μ g/mL and incubated at 27°C. After 4 days of incubation, the last blank in the serial dilution that had visible yeast growth was recorded as the titration end point and then was examined by subculture and microscopy. Results were analyzed with Student's *t* test.

Histology

Mice used for histologic examination were killed, and the eyes were enucleated at 1 day and 4 days p.i. Eyes from *C. albicans*-infected and mock-infected mice were formalin-fixed, paraffin-embedded, and sectioned at a thickness of 8 μ m for histologic study. Sections were

deparaffinized and stained with Grocott methenamine silver (GMS; Richard-Allan Scientific, Kalamazoo, MI) or periodic acid-Schiff (PAS; Sigma).

RESULTS

In Vitro Growth Kinetics of Wild-type *C. albicans* Strains

To determine whether inherent growth differences existed among the strains of *C. albicans*, the growth rates of strains VE175, B311, and SC5314 were evaluated in vitro. The overall growth kinetics of the three different strains was similar, with all three strains demonstrating similar lag, log-growth, and plateau phases (Fig. 1). Mock-inoculated negative control cultures remained negative for growth throughout the 7-day study. The results suggested that all three strains replicated at similar rates under controlled in vitro conditions and that no strain demonstrated an apparent growth advantage or disadvantage.

Visual Examination of Keratomycosis in Mice Infected with Wild-type *C. albicans* Strains

After the inoculation of 1×10^6 CFU of *C. albicans*, mice were visually evaluated daily for 8 days and scored for corneal involvement (Fig. 2). Mice infected with strain VE175 developed only mild keratomycosis by 24 hours p.i. that became significantly less severe by day 8 (day 1 vs. day 8; $P < 0.001$). Mice infected with strain B311 developed moderate keratomycosis on day 1 that persisted through day 4 (day 1 vs. day 4; $P = 0.7$) but that became less severe on and after day 5 (day 1 vs. day 5; $P = 0.04$). Mice infected with strain SC5314 also developed moderate keratomycosis, but corneal involvement remained moderate to severe through day 8 (day 1 vs. day 8; $P = 0.9$). As previously reported,¹¹ all mock-infected corneas had mild surface irregularities and swelling at 6 hours after the corneal scarification procedure but regained the normal appearance of a naive eye within 24 hours and then remained unchanged. Mice infected with strain B311 had significantly more disease

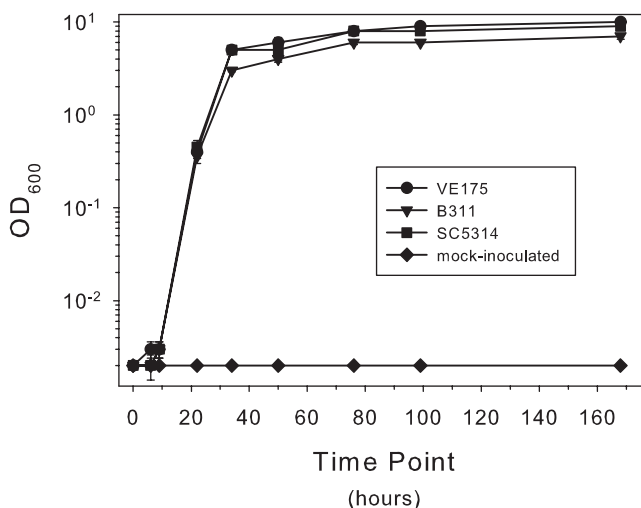


FIGURE 1. In vitro growth kinetics of *Candida albicans* strains VE175, B311, and SC5314. One thousand CFU of each cultured *Candida albicans* strain were inoculated into Sabouraud dextrose broth and monitored for growth for 7 days by optical density at a wavelength of 600 nm (OD₆₀₀). Mean OD₆₀₀ measurements (\pm SD) of six samples from two independent experiments are plotted. The lowest points plotted on the graph represent the optical density of the broth used as diluent and for blanking the spectrophotometer.

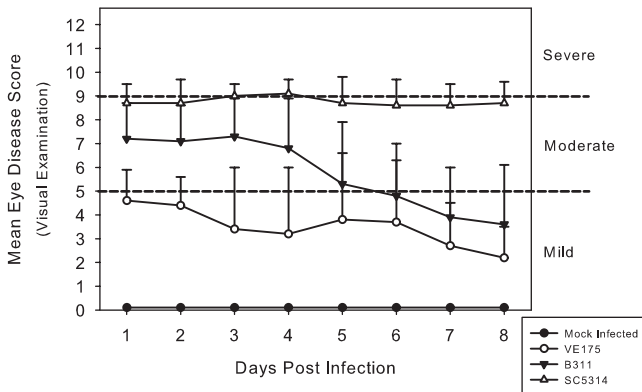


FIGURE 2. Severity of murine keratomycosis induced by *C. albicans* strains VE175, B311, and SC5314. Immunocompetent BALB/c mice were mock-infected or infected with 10^6 CFU of *C. albicans* and visually evaluated for their corneal involvement for 8 days. Mock-infected corneas received sterile PBS and served as controls. A total score less than 5 was categorized as mild disease, from 5 to 9 was considered moderate, and greater than 9 was severe. Each point represents the mean score (\pm SD) of 9 to 48 eyes.

than mice infected with strain VE175 from day 1 through day 4 ($P \leq 0.001$). However, disease with strain B311 improved from day 5 through day 8 and became similar to disease with strain VE175 ($P \geq 0.2$ for day 5 through day 8). Corneal disease with strain SC5314 was significantly greater than that caused by strain VE175 at all time points ($P \leq 0.001$). Representative findings from day 4 and day 8 are shown in Figure 3.

Quantitative Isolate Recovery of *C. albicans*

To correlate visible eye disease to fungal load within ocular tissues, mouse corneas were scarified and individually infected with 1×10^6 CFU of one of the three *C. albicans* strains or were mock-infected and analyzed by end point titration at 6 hours, 1 day, 4 days, and 8 days p.i. As shown in Figure 4, the rates of fungal clearance in mice were similar among the three strains at all time points evaluated ($P \geq 0.1$). Strains VE175, B311, and SC5314 were cleared from ocular tissues with significantly fewer organisms recovered from the infected corneas on day 8 compared with day 1 ($P \leq 0.009$ for all three strains). No *C. albicans* was recovered from mock-infected corneas at any time.

Histopathology of Mouse Eyes Infected with Wild-type *C. albicans* Strains

Histologic examination of infected eyes harvested 1 day and 4 days p.i. revealed invading yeasts in the corneal epithelium and the stroma. Corneas of infected animals showed epithelial thickening and varying degrees of stromal edema and inflammation. Massive hyphal formation was present in the cornea 1 day p.i. for strain SC5314 (Fig. 5A), but the yeast form was predominant 4 days p.i. The yeast form and some pseudohyphae were typically seen in VE175-infected animals, with the infection primarily more superficial and affecting the corneal epithelium and anterior portion of the stroma (Fig. 5B). Neutrophilic infiltration and edematous corneal stroma were prominent in corneas infected with strain SC5314, less severe with strain B311, and minimal with strain VE175 at 4 days p.i. Mock-infected eyes did not show signs of infection or inflam-

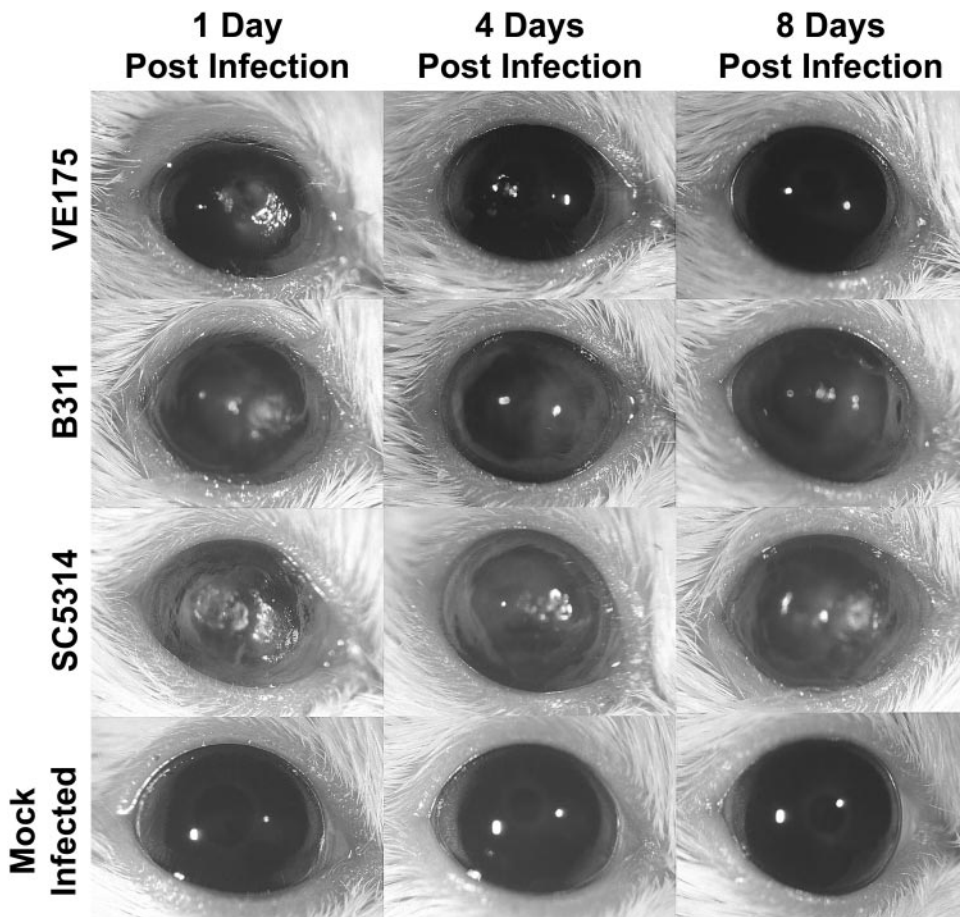


FIGURE 3. Clinical progression of murine keratomycosis induced by *C. albicans* strains VE175, B311, and SC5314. Corneas of immunocompetent BALB/c mice were mock-infected or infected with 10^6 CFU *C. albicans* and were photographed 1 day, 4 days, and 8 days after inoculation. Representative findings are shown.

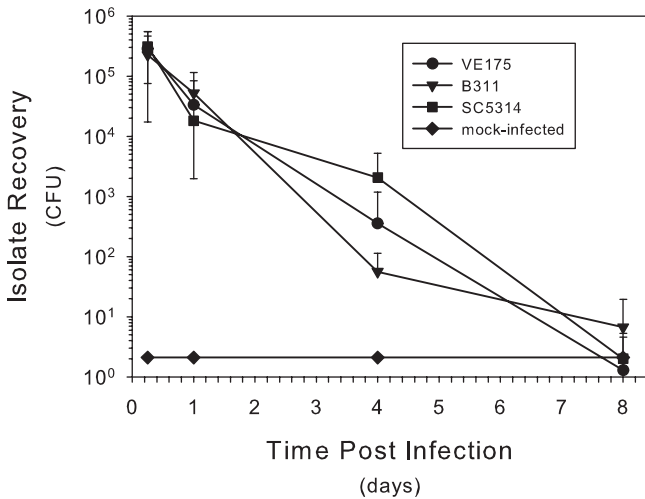


FIGURE 4. Quantitative isolate recovery of three different strains of *C. albicans* from infected corneas. Corneas of immunocompetent BALB/c were mock-infected or infected with 10^6 CFU of *C. albicans* strain VE175, B311, or SC5314 and analyzed by end point titration at 6 hours, 1 day, 4 days, and 8 days after inoculation. Mock-infected corneas received only sterile PBS. Culture-positive end points were converted to CFU, and the mean (\pm SD) of six eyes per time point are plotted. The lowest points plotted on the graph represent the lower limit of detection by the assay.

tion but did have residual microscopic epithelial changes from the initial scarification procedure.

Evaluation of Rim13p for *C. albicans* Virulence during Corneal Infection

To exclude any inherent growth differences for the homozygous *rim13*^{-/-} mutant Tn7-rim13 or its mutant-control reference strain DAY286, growth rates were evaluated in vitro and compared with those of SC5314. The overall growth kinetics of Tn7-rim13 and DAY286 were similar to those of SC5314, with no significant difference at any of the growth phases (Fig. 6). Mutant strains replicated at similar rates under controlled in vitro conditions, and no obvious growth advantage or disadvantage occurred compared with the wild-type strains used in the studies.

To determine the potential contribution of Rim101p-mediated filamentation on the virulence of *C. albicans* during corneal infection, Tn7-rim13 and DAY286 were compared with their progenitor wild-type strain SC5314. After inoculation of 1×10^6 CFU of each strain on the scarified corneas of immunocompetent mice, eyes were observed daily for 8 days and were scored for corneal involvement (Fig. 7). DAY286 induced severe keratitis indistinguishable from that caused by strain SC5314 throughout the 8 days of observation. Tn7-rim13 was fully attenuated, which caused only slight corneal opacity at day 1 that resolved by day 2 and remained clear through day 8. Differences in disease severity between Tn7-rim13 compared with SC5314 and DAY286 were significantly different ($P \leq 0.004$).

Histopathologic evaluation 1 day and 4 days p.i. of eyes from immunocompetent mice infected with 1×10^6 CFU of strains DAY286 and Tn7-rim13 correlated with disease severity (data not shown). Similar to SC5314, DAY286 infection resulted in invading yeasts in the corneal epithelium and the stroma, epithelial thickening, and varying degrees of stromal edema and inflammation. Neutrophilic infiltration and edematous corneal stroma were also prominent in corneas infected with strain DAY286. Hyphal formation was present in the

cornea 1 day p.i., but the yeast form was predominant at 4 days p.i. Eyes infected with Tn7-rim13 were essentially histologically normal except for an occasional adherent yeast form and mild epithelial changes from the initial scarification procedure.

Effect of Systemic Immunosuppression on Experimental *C. albicans* Keratitis

Immunosuppression increases the susceptibility of the host to infection and increases keratitis severity.^{11,34} To evaluate the effect of immunosuppression on the attenuated phenotype of the *rim13* mutant, immunocompetent (Fig. 8A) or cyclophosphamide-treated mice (Fig. 8B) were infected with 1×10^5 CFU of strains Tn7-rim13, DAY286, or SC5314 and were monitored for 8 days. SC5314 and DAY286 induced moderate keratitis in immunocompetent mice that began resolving on day 4 and subsided to mild disease on the final days of the study. At no time was disease severity between these two strains significantly different ($P \geq 0.13$). Eyes exposed to Tn7-rim13 remained disease free, which was significantly different from keratitis caused by SC5314 and DAY286 ($P \leq 0.01$). Pretreatment of mice with cyclophosphamide increased corneal disease severity, resulting in mean disease scores in the severe range for SC5314 and DAY286 at all time points during the 8-day evaluation. Immunosuppressive treatment partially restored virulence to strain Tn7-rim13, resulting in mean dis-

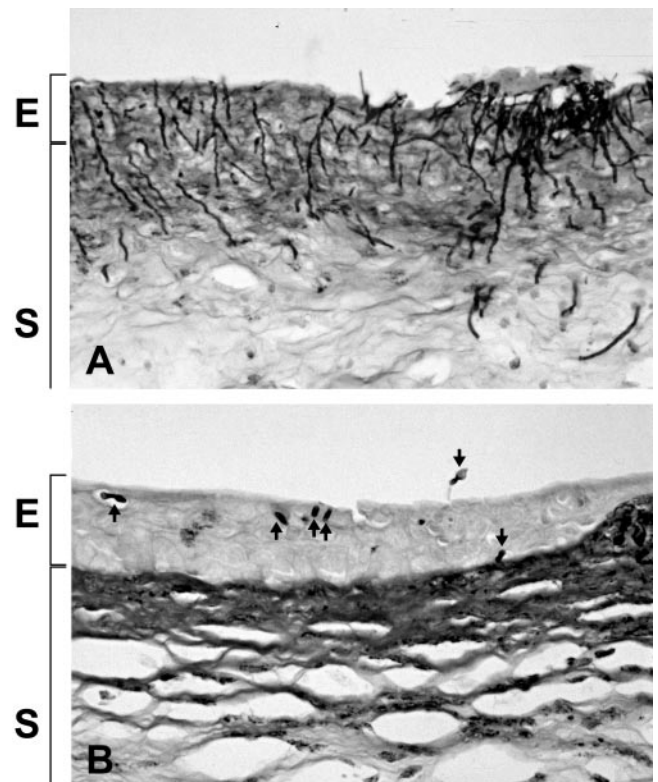


FIGURE 5. Histopathologic views of murine keratomycosis induced by *C. albicans* wild-type strains SC5314 and VE175. Mouse corneas were mock-infected or infected with 10^6 CFU of *C. albicans* and enucleated 1 day and 4 days later. Paraffin-embedded eyes were sectioned at a thickness of $8 \mu\text{m}$ and stained with PAS. Representative findings 1 day after infection are shown. (A) In SC5314-infected eyes, abundant fungi and corneal edema were evident in both the epithelium (E) and the corneal stroma (S). Pseudohyphae and hyphae were the predominant fungal forms. (B) VE175-infected eyes showed significantly fewer organisms and less edema and neutrophilic inflammation relative to SC5314-infected eyes. Budding yeasts (arrows) were the predominant fungal forms. Original magnification: (A) $\times 200$; (B) $\times 400$.

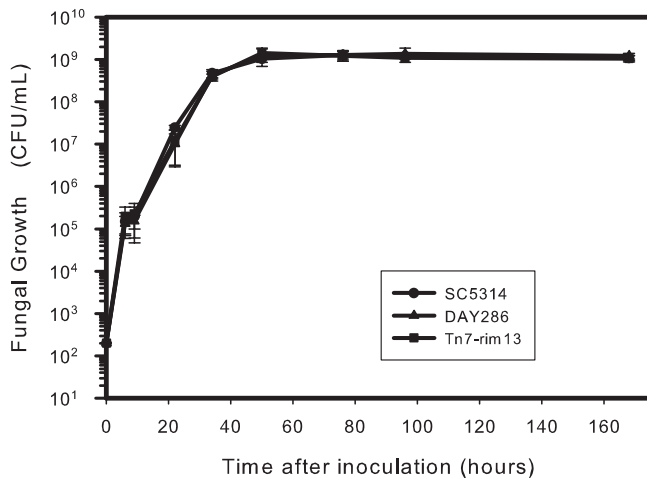


FIGURE 6. In vitro growth kinetics of *C. albicans* strains SC5314, Tn7-rim13, and DAY286. One thousand CFU of each cultured *C. albicans* strain were inoculated into Sabouraud dextrose broth and monitored for growth for 7 days. Optical density measurements were converted to CFU/mL, and the mean (\pm SD) of six samples from two independent experiments are plotted.

ease severity that increased to moderate levels, peaked at 3 days p.i., and resolved to mild levels with only slight corneal opacity. Even with the elevation in disease severity of cyclophosphamide-treated mice infected with Tn7-rim13, the mean degree of eye disease was significantly less than that observed for strains SC5314 and DAY286, except at day 3, the peak of corneal involvement ($P = 0.036$).

DISCUSSION

Candida keratitis is an opportunistic infection.³⁵ *C. albicans* can be acquired as the result of eye trauma or surgery, and it is an occasional contaminant encountered by corneal transplant recipients,³⁶ bandage contact lens wearers,³⁷ and patients with chronic ocular surface disease.³⁸ Experimental murine keratomycosis is a reliable mammalian system for understanding the pathogenesis of human ocular infection.¹¹ This model allows quantitative assessment of disease severity and is suitable for comparing virulence differences among fungal strains and mutants.

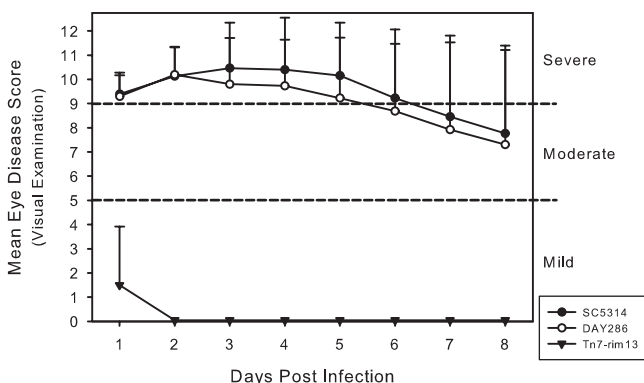


FIGURE 7. The severity of murine keratomycosis induced by *C. albicans* strains SC5314, DAY286, and Tn7-rim13 at 1×10^6 CFU. Immunocompetent BALB/c mice were mock-infected or infected with 10^6 CFU of *C. albicans* and evaluated for 8 days. Each point represents the mean score (\pm SD) of 5 to 18 eyes.

Specific virulence factors involved in *C. albicans* corneal infection are unknown but may be similar to mechanisms controlling candidal adherence and invasion during candidiasis of other organs and tissues.³⁹ Phenotypic switching—the ability of yeasts to form filamentous cells—is a crucial step in dissemination and infection. O'Day et al.²⁹ correlated *C. albicans* hyphal penetration with corneal disease severity in a rabbit model, and we recently reported an association between hyphal and pseudohyphal forms of *C. albicans* with more severe corneal disease in immunosuppressed mice.¹¹

In the present study we used a murine model to compare the corneal pathogenicity of three wild-type strains of *C. albicans*, a pH-mediated filamentation gene mutant, and a mutant reference strain control using clinical, microbiologic, and histopathologic methods. These *C. albicans* strains caused keratomycosis with different degrees of severity, suggesting that, as in systemic and other mucosal infections, *C. albicans* uses virulence factors during corneal infection.

Corneal disease caused by candidal strain SC5314 was significantly more severe than that caused by strain B311 or VE175. Histologic analysis further indicated that strain SC5314 was more invasive than the other two strains, with fungi penetrating more deeply into the corneal stroma. These results are consistent with previous results from the rabbit model.¹⁰ By the fourth day of experimental infection, the amounts of pseudohyphae, severe stromal edema, structural destruction,

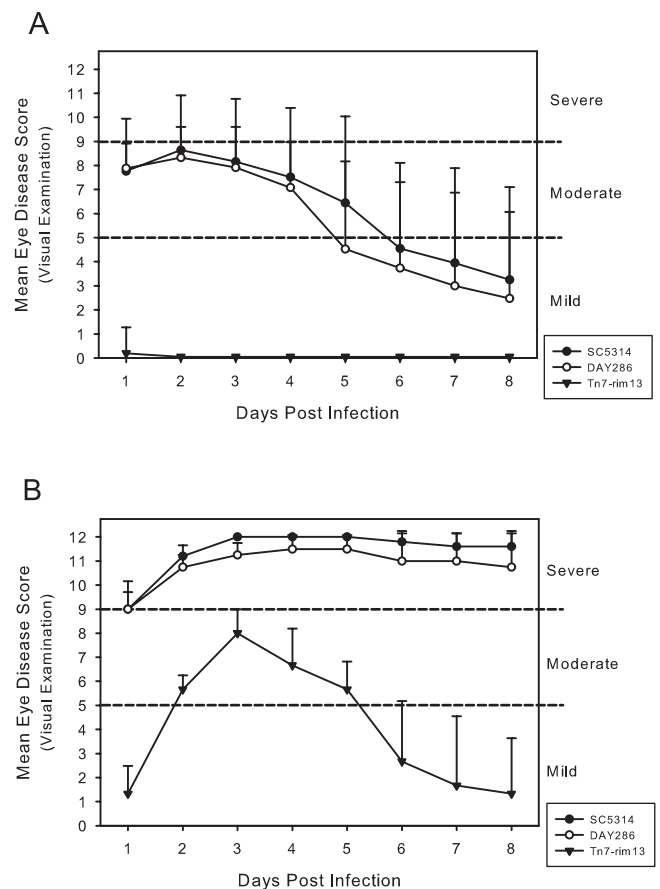


FIGURE 8. Severity of murine keratomycosis induced by *C. albicans* strains SC5314, DAY286, and Tn7-rim13 at 1×10^5 CFU. Immunocompetent BALB/c mice (A) or mice pretreated with cyclophosphamide (B) were mock-infected or infected with 10^5 CFU of *C. albicans* and evaluated for 8 days. Each point represents the mean score (\pm SD) of 17 to 35 eyes from immunocompetent mice and three to five eyes from cyclophosphamide-treated mice.

and inflammation were more evident in corneas infected with strain SC5314 than in those infected with strain B311 or VE175.

The greater virulence of strain SC5314 was not caused by an inherently accelerated rate of replication because all three strains had similar in vitro growth kinetics. Colonization factors mediating adherence are also unlikely to explain the corneal virulence of *C. albicans* because, at early stages of infection, similar numbers of viable organisms were recovered from infected corneas. Fungal clearance from the murine cornea was also similar for all three wild-type strains. This disparity between in vivo growth and clinical and histopathologic severity was also found in experimental *C. albicans* keratitis of rabbits.¹⁰ Our results and those of O'Day et al.²⁹ show that though the fungal load may be partially indicative of microbial replication and clearance, it is inadequate for predicting the disease severity or invasiveness of the infecting organism.

Based on previous reports^{28,29} and our current findings from studies using the wild-type strains of *C. albicans*, we speculate that the virulence factors involved in regulating phenotypic switching contribute to fungal penetration into the corneal stroma and account for differences in disease severity. Virulent strains SC5314 and B311 are induced to filament in vitro by growth at pH 8, but the less virulent strain VE175 fails to filament (data not shown). A shifting corneal microenvironment around colonizing yeasts could induce the candidal Rim101 pathway.⁴⁰ Genetically regulated fungal filamentation would facilitate stromal invasion and trigger an inflammatory response.⁴¹

We previously showed that host factors, including immune status and genetic background, contribute to the outcome of murine keratomycosis caused by *C. albicans*¹¹ and *Fusarium solani*.²⁸ Our current results are an extension of these findings. Pretreatment of mice with cyclophosphamide resulted in more severe disease for wild-type and mutant strains of *C. albicans*. Immunosuppressive treatment restored virulence partially, but not completely, to the *rim13* mutant, suggesting that host factors and infecting pathogen factors influence disease profile.

Our current findings demonstrate that various strains of *C. albicans* have inherent differences in pathogenic potential. Furthermore, our studies using a homozygous *rim13* mutant provide direct evidence that filamentation is important for *C. albicans* virulence during corneal infection and indicate that a hyphal morphogenesis-related gene is a virulence factor for fungal keratitis. The pronounced attenuation observed for strain Tn7-rim13 is consistent with the role of fungal filamentation in candidiasis and supports the concept of developing therapeutic approaches targeting the Rim101 signal transduction pathway. The experimental murine keratomycosis model will be useful for continued investigation of virulence factors mediating fungal infection of the eye.

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