

Differences in Virulence between Two *Candida albicans* Strains in Experimental Keratitis

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PURPOSE. To study the differences in disease caused by two wild-type strains of *Candida albicans* in a model of contact lens-facilitated keratitis in rabbits.

METHODS. Two strains, SC5314 and VE175, were examined. Standardized inocula were placed on the debrided corneal surface of one eye in Dutch belted rabbits and covered with a contact lens. A temporary tarsorrhaphy was opened after 24 hours with removal of the contact lens. Six days later, corneas were photographed and animals killed. Corneas were bisected with one half for quantitative isolate recovery and the other for stromal penetration by hyphae.

RESULTS. Strain SC5314 was significantly more virulent. The mean hyphal penetration into the cornea was $24.4\% \pm 8.5\%$ of the corneal thickness, and in three of six corneas hyphae penetrated through the entire cornea. In contrast, for VE175, the mean hyphal penetration was $2.6\% \pm 1.2\%$. The difference between these two strains was statistically significant ($P = 0.0297$). Hyphae did not penetrate into the deep layers of the cornea in any of the six rabbits infected with VE175. The grading of clinical disease was consistent with histology, in that strain SC5314 caused more severe infection than VE175 and the difference was statistically significant ($P = 0.0048$). There was no difference in isolate recovery.

CONCLUSIONS. Wild-type strains of *C. albicans* can differ significantly in virulence as measured by depth of fungal invasion into corneas and clinical evaluation of infection. Further characterization of the intrinsic genetic differences between such strains may help identify factors responsible for fungal virulence. (*Invest Ophthalmol Vis Sci.* 2000;41:1116-1121)

In recent years, research on fungal infection in the eye has centered on pharmacologic interventions using existing antifungal drugs.¹⁻⁹ These drugs have many limitations, not the least of which are toxicity and selection of resistant strains in vivo. To gain insights in the development of new pharmacologic approaches, an understanding of pathogenic mechanisms and factors controlling fungal virulence is essential. With recent advances in knowledge of the molecular events surrounding fungal infection and an enhanced ability to manipulate fungal genes, the opportunity now exists to begin to investigate novel ways to block or retard fungal mechanisms controlling virulence. In this regard, an important first step is to reliably identify fungal strains that exhibit different degrees of virulence in vivo. Further genetic and molecular characterization of such strains may shed new light on the mechanisms of fungal virulence. In this article, we report the clinical, mycologic, and pathologic characterization of two wild-type

Candida albicans strains, with markedly different degrees of virulence in an animal model of *C. albicans* keratitis.

MATERIALS AND METHODS

Strains

We evaluated two wild-type strains of *C. albicans*. These strains have both been used previously and reported in the literature. Strain VE175 is a human corneal isolate that we have used extensively in previous models of fungal keratitis in rabbits.^{1,3,5,7,10-15} Studies with SC5314 were originally reported by Gillium et al. in 1984.¹⁶ SC5314 has been used extensively for genetic studies of *C. albicans*.¹⁷⁻³¹ Both these strains were maintained at -70°C in yeast extract-peptone-dextrose broth supplemented with 16.7% glycerol.

Inoculum

For these experiments, both strains were cultured overnight in yeast extract-peptone-dextrose broth at 30°C on a rotary shaker at 200 rpm. We used 25 ml of media in a 50-ml sterile polypropylene tube. Just before inoculation the cultures were centrifuged at 2500g for 3 minutes, the supernatant was poured off, and the tubes were inverted and allowed to drain. The yeast pellet was used to inoculate the rabbit's corneas.

Animals

Dutch belted rabbits of either sex weighing 1 to 2 kg were used in these experiments. All animals were treated in accordance

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with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Model of Invasive Keratitis

Dutch belted rabbits were anesthetized with intramuscular ketamine and xylazine.³² Corneal anesthesia was obtained with topical 0.5% proparacaine hydrochloride. The nictitating membrane was removed by sharp dissection. A 7-ml disc of epithelium was marked by gentle pressure with a corneal trephine and completely removed by atraumatic scraping with a 15 Bard-Parker blade. An inoculum of 100 μ l, containing approximately 2×10^8 blastoconidia, was placed on the denuded cornea and covered with a flexible contact lens made from etafilcon A (Acuvue; Johnson and Johnson Vision Products, Jacksonville, FL). A tarsorrhaphy prevented extrusion of the contact lens. The tarsorrhaphy sutures and contact lens were removed 24 hours later.

Clinical Evaluation

Before death, the infected eyes of all rabbits were photographed. Three observers, masked to the identity of the individual rabbits, examined these photographs and scored the severity of the infection as follows: grade 0, no infection; grade 1, less than 25% of the central 7 mm (debrided section) of the cornea involved; grade 2, 25% and 50% of the central 7 mm of the cornea involved; grade 3, 50% to 75% of the central cornea involved; grade 4, 75% and 100% of the central cornea involved; and grade 5, any amount of corneal involvement with a hypopyon present.

The observers also ranked all twelve rabbits from 1 to 12 based on severity of corneal disease with 1 representing the least infected cornea and 12 the most severely infected cornea. For this ranking, no ties were allowed.

Postmortem Tissue Dissection

Rabbits were killed by rapid intravenous injection of a 26% pentobarbital sodium euthanasia solution (Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA). The infected corneas were removed at the limbus and bisected longitudinally. One half was cut into small pieces for quantitative isolate recovery, and the other half was fixed in formalin for histology.

Quantitative Isolate Recovery of Fungi from Infected Corneas

Corneal halves were placed individually into 3-ml sterile normal saline in a test tube. They were then processed for quantitative isolate recovery using our standard method of grinding the cornea and plating 10 or 100 μ l samples of serial dilutions, in triplicate, on Sabouraud's agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD). The colonies were then counted, and the number of colony-forming units recovered from each corneal half was calculated.^{3,7}

Histology

In previous studies with strain VE175 we have demonstrated a very high correlation between data derived from a single section taken from the central cornea and a series of sections throughout the cornea.³³ Examination of three sections from each rabbit in this experiment, spaced at 100 μ m from the center of the corneal lesion, showed no gross differences between rabbits for either strain. Therefore, in this experiment

we studied a single corneal section in detail for each rabbit. This 5- μ m section was cut from the midcornea, along the edge of the embedded corneal half. The section was stained with periodic acid-Schiff's reagent, and counter-stained with fast green stain.

Hyphal Penetration

Pictures were taken of one entire corneal section from each infected cornea with an exposure control unit (model PM-20; Olympus, Lake Success NY) attached to a microscope (model BX-40; Olympus). The final magnification of $\times 150$ allowed us to determine and mark the deepest point of corneal penetration of individual hyphae. We then measured the corneal thickness and hyphal length for each observed hypha with a ruler and calculated the depth of penetration as a percentage of corneal thickness. As a measure of the total fungal invasion for each cornea, the fungal biomass was calculated by summing the hyphal penetration measurements for each cornea. All observations were made by masked observers.

Statistics

Standard analysis of variance methods were used to compare the two groups of rabbits. A log transformation of the isolate recovery rate was performed. The numbers of hyphae and the percentage invasion of the cornea by the hyphae were compared directly without transformation. These parametric data were analyzed using a general linear model procedure. Because there were only six rabbits in each group, the parametric data, as well as the nonparametric data derived from the clinical scores and clinical ranks, were analyzed using the Wilcoxon two-sample test with continuity correction. Statistical analyses were performed using commercial software (SAS for Windows; SAS Institute, Cary, NC).

RESULTS

Clinical Evaluation

As shown in Table 1 and clearly shown in Figures 1A and 1B, there was a statistically significant difference in the severity of disease caused by the two strains as measured by clinical scoring of disease and clinical ranking within this experiment by all three observers. Strain SC5314 (Fig. 1A) caused significantly more severe disease than strain VE175 (Fig. 1B).

Isolate Recovery

According to a standard quantitative isolate recovery technique,³ there was no statistical significance in isolate recovery between the two strains, either when compared parametrically (Table 2) or when the recovery rates were ranked as shown in Table 3.

Hyphal Invasion

There was a marked difference in hyphal invasion between the two strains. Six days after inoculation, strain VE175 was largely confined to the anterior corneal stroma (Fig. 1D). The mean median depth of penetration into the cornea was only 2.6% of the corneal thickness, and the mean maximum depth of penetration was only 3.1%. By contrast, strain SC5314 showed a marked propensity for deep corneal invasion (Fig. 1C) with the mean depth of penetration at 24.4% of the corneal thickness.

TABLE 1. Clinical Evaluation of Rabbits with *Candida albicans* Keratitis

Strain	<i>n</i>	Observer 1		Observer 2		Observer 3		All Observers	
		Mean Clinical Score	Mean Rank Score						
SC5314	6	3.3 ± 0.4	9.5 ± 0.8	2.5 ± 0.6	9.3 ± 0.9	2.2 ± 0.3	9.5 ± 0.8	2.7 ± 0.4	9.4 ± 0.8
VE175	6	1.0 ± 0.3	3.5 ± 0.8	0.8 ± 0.3	3.7 ± 0.9	0.8 ± 0.2	3.5 ± 0.8	0.9 ± 0.2	3.6 ± 0.8
<i>P</i>		0.0052	0.0051	0.0451	0.0082	0.0096	0.0051	0.0048	0.0051

Values are means ± SEM. *n* is number of rabbits. Mean clinical score is based on a 0–5 grading scale as described in the Methods section. Mean rank score is 1–16, with 1 being the least infected rabbit and 16 being the most infected rabbit.

Measurement of the degree of penetration was hampered somewhat by the presence of edema in the area of the infection. Nonetheless, the difference between the infecting strains was so marked that it could have been assessed without actually measuring the depth of hyphal penetration. One of six corneas with each strain failed to demonstrate any fungal invasion for the histologic section that we measured in detail. Histologically, the periphery of the area of inoculation around the trephine mark often showed abnormally deep penetration that was not consistent with the rest of the lesion. This was

probably due to a cut through the anterior stroma caused by the trephine. This artifact was ignored in making the measurements.

The high magnification histology (Figs. 1E, 1F) demonstrates that during corneal invasion the morphology of the two strains was different. Strain VE175 invaded the cornea in a pseudohyphal form characterized by the pinching of the cell wall between cells at the septae. Strain SC5314 invaded the cornea in what appeared to be a true hyphal phase.

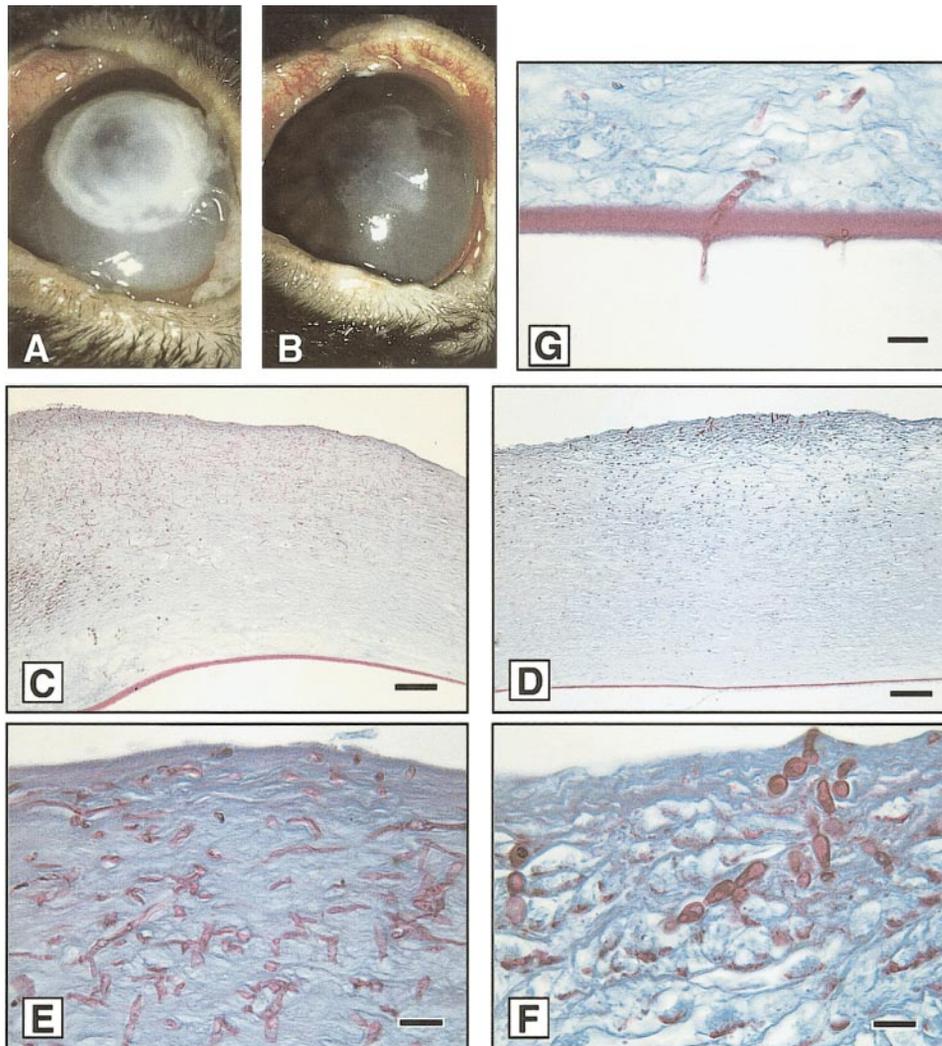


FIGURE 1. Clinical photographs of rabbits with *C. albicans* keratitis strain SC5314 (A) and strain VE175 (B). (C, D) Photomicrographs of full-thickness corneal sections from rabbits shown in photographs (A) and (B). (C) Section from rabbit infected with strain SC5314. Note hyphal invasion into the deep stroma. (D) Photomicrograph of corneal section infected with strain VE175. Note that fungal invasion is limited to the superficial cornea. (E) Oil immersion photomicrographs of strain SC5314 invading the cornea. These fungal elements appear to be true hyphae. (F) Oil immersion photomicrograph of strain VE175 invading the cornea. These fungal elements appear to be pseudohyphae. (G) Hypha of strain SC5314 penetrating Descemet's membrane and invading the anterior chamber of the rabbit eye. All sections were stained with PAS and counterstained with fast green. Bar, (C, D) 100 μ m; (E, F) 10 μ m.

TABLE 2. Isolate Recovery and Hyphal Penetration into the Cornea: Parametric Data

Strain	n*	L ₂ CFU†	Hyphae/Section‡	Biomass§	Hyphal Penetration into the Cornea		
					Mean	75% Quantile	Maximum
SC5314	6	13.89 ± 0.79	121.5 ± 93.3	3348.8 ± 2166.4	24.4% ± 8.5%	33.6% ± 10.6%	52.6% ± 16.1%
VE175	6	12.88 ± 0.27	4.3 ± 3.9	17.6 ± 15.3	2.6% ± 1.2%	2.8% ± 1.3%	3.1% ± 1.4%
P		0.2572	0.2379	0.1552	0.0297	0.0163	0.0119

Values are means ± SEM.

* Number of rabbits.

† Log base 2 isolate recovery per half cornea.

‡ Number of hyphae per corneal histologic section.

§ Sum of hyphal penetration measurements.

|| Depth of hyphal penetration into the cornea expressed as a percentage of corneal thickness.

After detailed analysis was completed for a single section from each cornea, multiple serial sections for all corneas were scanned for evidence of complete corneal penetration. For strain SC5314, the hyphal penetration was 100% with fungi crossing Descemet's membrane into the anterior chamber in three of the six corneas (Fig. 1 G). For strain VE175, the fungus invaded farther than half the corneal thickness in only one of the six corneas and did not reach Descemet's membrane in any cornea.

DISCUSSION

The model of infection used in these experiments was developed in our laboratory in an effort to parallel as closely as possible the circumstances surrounding human infection. Several important features appear to make this model more useful than previous attempts.

First, the cornea is not traumatized other than by removal of the epithelium so that spores are not inoculated into the stroma. As we observed in these animals, when the organism is inoculated into the cornea, as occurred in several rabbits when the trephine cuts penetrated the stroma, the whole dynamic of the infection is changed. Because of this observation, we no longer use a trephine to mark the epithelium but instead use a filter paper disc moistened with *N*-heptanol to remove the epithelium from the cornea.

Second, in our previous models of corneal infection, blastoconidia were injected directly into the corneal stroma. Because some but not all of the blastoconidia germinate, viable fungus is present in the stroma in both growing and resting

phases. This leads to confusion of the evaluation of disease by both histology and quantitative isolate recovery.

In this model, organisms invading the cornea are only in the growing phase, mimicking human infection. Blastoconidia that do not attach and invade the cornea appear to be swept away from the corneal surface, probably by the mechanical action of the lids.

Third, the host animal is immunocompetent. We have not found it necessary to use local injection of corticosteroid to initiate infection. With this model, we are able to detect differences in virulence between strains that are capable of establishing disease in the rabbit cornea. Of the three measures used, clinical photography, histologic assessment and quantitative mycology, only quantitative isolate recovery failed to demonstrate a difference between these two strains. The histologic parameters chosen (number of hyphae per section, biomass, and depth of invasion into the cornea) clearly demonstrate aspects of virulence that differ between the two strains. These measurements, although time consuming and tedious, are straightforward to obtain because fungal elements are clearly visible under the microscope, with appropriate staining. Alterations in corneal thickness, as a result of the infection or as a fixation artifact, add some uncertainty to the assessment of depth penetration but the difference in invasiveness between the these strains was sufficiently great to render dependence on precise measurements unnecessary. Although we did not study them, other objective histologic measures deserve further investigation, including measurement of horizontal spread of the fungus through the cornea, and the severity of the host response.

TABLE 3. Isolate Recovery and Hyphal Penetration into the Cornea: Rank Data

Strain	n*	L ₂ CFU†	Hyphae/Section‡	Biomass§	Hyphal Penetration into the Cornea		
					Mean	75% Quantile	Maximum
SC5314	6	7.5 ± 1.8	9.0 ± 1.0	9.2 ± 0.9	9.5 ± 0.8	9.5 ± 0.8	9.5 ± 0.8
VE175	6	5.5 ± 1.1	4.0 ± 1.1	3.8 ± 1.0	3.5 ± 0.7	3.5 ± 0.7	3.5 ± 0.7
P		0.3611	0.0070	0.0029	0.0002	0.0002	0.0002

Values are mean ranks ± SEM.

* Number of rabbits.

† Log base 2 isolate recovery per half cornea.

‡ Number of hyphae per corneal histologic section.

§ Sum of hyphal penetration measurements.

|| Depth of hyphal penetration into the cornea expressed as a percentage of corneal thickness.

Histologic examination of multiple sections from infected corneas is extremely labor intensive. In previous work, we have shown that analyses of one to three sections, separated by 100 μm , from an infected cornea correlate well with data from multiple sections throughout the cornea.³³ This greatly reduces the amount of work involved in each experiment.

Clinical evaluation by grading of photographs of infected corneas produced the same result. Although the scoring method was empirical, it correlated with a severity ranking of the disease including all 12 rabbits for the three masked observers. The failure to detect a difference between the two strains by quantitative isolate recovery was not unexpected, given the inability of this method to differentiate between groups of rabbits with clearly different clinical disease that we have observed in other studies.³³ The reason for this insensitivity seems to be the varied colony-forming potential of hyphal fragments of unequal length or cell numbers. Quantitative isolate recovery appears to be most suited to assessment of unicellular phase organisms, particularly when subtle differences are present. For these studies, we endeavored to control for experimental factors that might influence virulence. Each strain was maintained in an identical manner, the preparation of the inoculum was identical, and the concentration of the inoculum paste, although not the same, was similar. Only a small percentage of the inoculum attaches to the cornea and is not eliminated when the tarsorrhaphy sutures and contact lens are removed on the day after inoculation. In previous experiments we have shown that the keratitis caused by *C. albicans* in Dutch belted rabbits is remarkably consistent from animal to animal, and for this reason this animal species was selected for these studies.³⁴ All observations were made in a masked fashion. Thus, although we cannot exclude extraneous factors that could influence the degree of penetration, and the actual measurements have an element of uncertainty due to edema of the cornea that occurs with the infection, all the evidence from these studies points to an intrinsic difference between these strains to account for the difference in virulence.

Various intrinsic differences between the *C. albicans* strains can be responsible for the observed marked difference in pathogenicity in vivo. First, fungal genes that control morphogenesis may be involved. Recent work in the signal transduction pathways that lead to hyphal or yeast formation in *C. albicans* demonstrated that genetic mutations in key virulence factors of the fungus can have profound effect on fungal virulence.^{21,24} For example, Lo et al.²⁴ showed that null mutations in proteins such as CPH and EFG lead to a locked morphologic state of the fungus in the presumed less virulent yeast form. On the contrary, Braun and Johnson²¹ demonstrated that knockout deletion mutations in transcriptional repressors such as TUP1 could in fact lock the organism in the presumed more invasive hyphal form. It should be cautioned that the relationship between dimorphism and virulence in *C. albicans* has not been well established, evidenced by our recent study that the TUP1 homozygous deletion mutant is in fact less invasive than the isogenic strain in our model of contact lens-facilitated keratitis in rabbits.³⁵ In the present work, it is interesting to note that the less virulent strain, VE175, was unable to form true hyphae. It appears, therefore, that although the relationship between dimorphism and *C. albicans* pathogenicity is not well established, *C. albicans* strains that fail to form true hyphae in vivo are perhaps less penetrative, and thus cause less disease in tissues such as the

cornea. In addition to the investigation of genes controlling *C. albicans* dimorphism, other genes that are involved in fungal adherence and penetration can also be involved in fungal virulence.³⁶⁻³⁹

In summary, we believe the present work demonstrates that intrinsic differences between *C. albicans* wild-type strains can lead to marked difference in predilection toward clinical infection. Further investigation in the molecular mechanisms responsible for this difference may shed new light on the pathogenic mechanisms of this fungus and suggest new therapeutic targets.

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