Immunosuppression Affects the Severity of Experimental *Fusarium solani* Keratitis

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We have established a mouse model of corneal fusariosis that permits the evaluation of fungal infection and pathogenesis. Corneas of immunocompetent and cyclophosphamide-treated adult BALB/c mice were topically inoculated with *Fusarium solani* after corneal scarification. Eyes were scored for corneal involvement daily for 8 days and at 2 weeks after infection. Eyes were enucleated at various time points for quantitative fungal recovery and histopathological examination. An inoculum-dose response was observed in cyclophosphamide-treated mice, and fungi were recovered from the infected eyes by quantitative microbial culturing. Treatment with cyclophosphamide increased disease severity and delayed fungal clearance. Fungal hyphae, inflammatory cells, and stromal edema were histologically evident within corneal tissue and correlated with disease severity. Although the mouse cornea resists fungal infections, *F. solani* keratitis could be induced in immunosuppressed mice after surface scarification, which resulted in infection and clinical disease that could be evaluated both in vivo and in vitro.

*Fusarium* is a genus of ascomycetes that is widely distributed in soil and on vegetation [1]. Besides being a common plant pathogen and potential mycobicide [2], *Fusarium* species cause opportunistic mycosis in humans, including superficial fusariosis that occurs after trauma and systemic infection during immunosuppression [3]. Of >20 species, *Fusarium solani* is among the most pathogenic [4]. Once thought to be a relatively uncommon cause of ocular disease [5, 6], *Fusarium* species have emerged as one of the leading causes of human keratomycosis [7] and are, along with *Aspergillus* and *Candida* species, among the most common fungal corneal isolates in the southern United States, South America, the Middle East, sub-Saharan Africa, India, and southeast Asia [8–10].

Because of the complex life cycle of fungi, understanding the pathogenesis of keratomycosis requires animal models that allow high reproducibility and sensitive quantitation. Experimental fusariosis of the cornea is generally mild and self-limiting in immunocompetent animals, and the consistent induction of corneal fusariosis in animals frequently requires immunosuppression by treatment with corticosteroids [11–13]. Animal models of fusarial keratomycosis have been produced in both rabbits [14–22] and rats [23]. These models have been used to assess diagnostic [24–26] and therapeutic [27, 28] strategies and to attempt to understand the pathogenesis of fungal corneal disease [29, 30]. Despite these advances, the pathogenic mechanisms of oculomycosis remain largely undefined.

The focus of the present study was to develop a mouse model of corneal fusariosis that is comparable to human disease and would be amendable to genetic and immunological manipulation. A mouse model using corneal surface inoculation, rather than intrastromal injection, would allow studies of fungal adherence and invasion and more closely mimic the majority of natural eye infections in humans. We examined the
kinetics of corneal infection by *F. solani* in inbred mice, with and without immunosuppressive treatment. This mouse model resulted in relevant clinical disease, with the severity of corneal fusariosis being immune-status and inoculum-dose dependent.

**MATERIALS AND METHODS**

**Fungal culture.** The strain of *F. solani* used for these studies was SRL-F2 (Sid W. Richardson Ocular Microbiology Laboratory Culture Collection, Baylor College of Medicine, Houston, TX), a clinical human isolate obtained from corneal scraping. Speciation was based on macroscopic and microscopic criteria [31]. A 1-mL homogenate of cultured SRL-F2 was quantified with a spectrophotometer, to obtain an optical density reading at 600 nm, and then 2-fold serially diluted in Sabouraud dextrose broth (Difco) that contained chloramphenicol (Sigma). After 7 days of incubation at 35°C, the last blank in the serial dilution that had visible fungal growth was recorded as the titration end point and was examined by subculturing and microscopy. The number of culturable units (CU) empirically obtained by end-point titration was compared with the initial optical density reading at 600 nm, yielding 1 OD<sub>600</sub> unit as equivalent to (5.3 ± 2.7) × 10<sup>3</sup> CU. For corneal inoculation, SRL-F2 was grown on Sabouraud dextrose agar (Difco) for 4 days at 35°C, harvested in sterile PBS, and diluted with sterile saline, to yield 1 × 10<sup>5</sup>, 1 × 10<sup>4</sup>, or 1 × 10<sup>3</sup> CU/5–μL inoculum.

**Animals.** Adult female BALB/c mice (Harlan Sprague-Dawley) were used at 6–8 weeks of age (weight, ~17–20 g). Mice were housed in accordance with National Institutes of Health guidelines, and all procedures in the study conformed to the *Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research* and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Untreated immunocompetent mice and mice pretreated with cyclophosphamide (Sigma) were both used in these studies. An intraperitoneal injection of cyclophosphamide at 180 mg/kg body weight was given 5, 3, and 1 day before corneal inoculation, a regimen that has been previously demonstrated to induce generalized immunosuppression for ≥2 weeks after treatment and to have an immunosuppressive effect in ocular tissue [32–34].

Mice were anesthetized by the intramuscular administration of a rodent combination anesthetic that contained ketamine, xylazine, and acepromazine [33]. The cornea of the right eye of each mouse was scarified by use of a hypodermic needle, to create a superficial wound of intersecting marks in a grid pattern, as described elsewhere [33]. The animals received a 5-μL inoculum of *F. solani* applied to their scarified corneas. The eyelids were rubbed together for 10 s, to evenly distribute the inoculum over the corneal surface. Animals that served as negative controls were mock inoculated with the carrier (sterile PBS). All mice were scored daily for corneal involvement, for the first 8 days and at 14 days after infection. Groups of mice were killed and eyes enucleated 6 h and 1, 4, 8, and 14 days after infection and were processed for quantitative microbial cultures. Additional eyes were harvested 1, 4, and 8 days after infection and were processed for histopathological examination.

**Clinical scoring.** The severity of keratomycosis in the animals was visually scored with the aid of a dissecting microscope and slit lamps [33, 35]. Because the eyes were scored for corneal involvement for all animals, the sample size of earlier time points were larger than those at later time points. A grade of 0–4 was assigned to each of 3 criteria: area of corneal opacity, density of corneal opacity, and corneal surface regularity, as detailed in table 1 and described elsewhere [33]. Scores from all 3 categories for each eye were tallied daily, to yield a possible total score of 0–12. Results were evaluated for statistical significance by use of a Kruskal-Wallis 1-way analysis of variance on ranks. Pairwise multiple comparison procedures included Dunn’s method and the Tukey test. A total score of 1–4 was categorized as mild eye disease, a score of 4–8 was categorized as moderate eye disease, and a score of 8–12 was categorized as severe eye disease.

**Quantitative fungal recovery.** The culturable fungal load in ocular tissue was quantitated by use of an end-point culture assay [33]. In brief, *F. solani*-infected and mock-infected eyes were enucleated and individually ground in frosted-glass grinders, with 1 mL of Sabouraud dextrose broth that contained 50 μg/mL chloramphenicol. A 0.5-μL homogenate aliquot was 4-fold serially diluted in Sabouraud dextrose broth that contained chloramphenicol and was incubated at 35°C. After 7 days of incubation, the last blank in the dilution series that had visible fungal growth was recorded as the titration end point. Results

### Table 1. Quantitative scoring system for mouse fusarial keratitis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Grade of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of corneal opacity, %</td>
<td>1–25</td>
</tr>
<tr>
<td>Density of corneal opacity</td>
<td>26–50</td>
</tr>
<tr>
<td>Surface regularity</td>
<td>51–75</td>
</tr>
<tr>
<td></td>
<td>76–100</td>
</tr>
<tr>
<td>Slight surface irregularity</td>
<td>Slight surface irregularity</td>
</tr>
<tr>
<td>Cloudy but outline of iris and pupil remain visible</td>
<td>Cloudy, opacity not uniform</td>
</tr>
<tr>
<td>Rough surface, some swelling</td>
<td>Significant swelling, crater or serious descemetocele formation</td>
</tr>
<tr>
<td>Perforation or descemetocele formation</td>
<td></td>
</tr>
</tbody>
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Inoculation dosage. The virulence of *F. solani* strain SRL-F2 was initially evaluated in mice that had been immunosuppressed with cyclophosphamide. The severity of disease demonstrated a relative dose response to the infecting inocula (figure 1). Mice infected with $1 \times 10^3$ CU developed only mild keratomycosis by 24 h after infection that persisted for the 8 days of evaluation. Mice infected with $1 \times 10^4$ CU developed mild keratomycosis on day 1 that became significantly worse on day 3 ($P = .03$, day 1 vs. 3). This infection progressed, with severe inflammation developing by day 4 that persisted through day 8. Mice infected with $1 \times 10^5$ CU developed moderate keratitis within 1 day that progressed to severe inflammation by day 3, which persisted through day 8. None of mock-infected corneas developed any visible opacity. Keratitis was significantly more severe in mice infected with $1 \times 10^5$ CU than in those infected with $1 \times 10^4$ CU ($P < .03$) or infected with $1 \times 10^4$ CU on days 3–8 ($P < .001$). Inoculation dose response of the severity of keratitis in immunosuppressed mice. Cyclophosphamide-treated BALB/c mice were mock infected or infected with $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ culturable units (CU) of *F. solani* and evaluated for keratitis severity over the course of 8 days. Mock-infected corneas received only carrier (sterile PBS) and served as controls. A possible score of 0–12 was assigned daily for each eye, relative to corneal involvement and disease severity. Each bar represents the mean score (±SD) of 6–18 eyes.

were analyzed statistically by use of Student’s *t* test. The endpoint cultures were confirmed by subculturing and microscopic examination.

**Histological testing.** *F. solani*–infected and mock-infected eyes were fixed in formalin, embedded in paraffin, and serially sectioned at a thickness of 5 μm for histological study. The sections were deparaffinized, stained with periodic acid–Schiff (Sigma), and examined microscopically.

**RESULTS**

**Inoculation dosage.** Consistent with the results of previous mouse models that used corneal scarification [33, 35, 36], mock-infected corneas presented mild surface irregularities and swelling 6 h after the corneal scarification procedure but regained the normal appearance of a naive eye within 24 h and subsequently remained unchanged, regardless of immune status. In infected mice, the overall kinetics of the disease was different between immunocompetent and immunosuppressed mice (figure 2). Immunocompetent mice developed mild keratomycosis by 24 h after infection that began to resolve by day 2. Seventy-one percent (10/14) of these eyes regained a normal appearance after day 4. In contrast, cyclophosphamide-treated mice developed mild to moderate keratomycosis initially during the first 3 days, and their corneal involvement progressed to severe inflammation by day 4 that persisted through day 8. On day 14 after infection, keratomycosis in the immunosuppressed mice resolved significantly, to a moderate level ($P = .01$, day 8 vs. 14). The mean corneal disease was more advanced in the cyclophosphamide-treated mice than in the immunocompetent mice, from 2 days through 2 weeks after infection, with the difference being significant on days 3–8 ($P = .996$ for day 1, $P = .252$ for day 2, and $P < .001$ for days 3–8). Representative findings from days 1, 4, and 8 after infection are shown in figure 3. **Fungal recovery.** As shown in figure 4, *F. solani* was cleared from the ocular tissues of both immunocompetent and immunosuppressed mice, with significantly fewer organisms recovered from the infected corneas day 14 after infection than at 6 h after infection ($P = .04$). Fungal clearance occurred sooner in immunocompetent mice than in cyclophosphamide-treated mice. Significantly fewer organisms were recovered from the corneas of immunocompetent mice on days 1, 4, 8, and 14 after infection than at 6 h after infection ($P = .003$). In...
contrast to the clearance of *F. solani* from the infected eyes of immunocompetent mice beginning within 1 day after infection, the reduction in fungal load in immunosuppressed mice was delayed. A significant reduction in the number of fungi from the corneas of cyclophosphamide-treated mice, compared with that at 6 h after infection, was observed only on days 8 and 14 after infection (*P* = .03). As expected, *F. solani* was not recovered from mock-infected corneas at any time.

**Histopathological testing.** The histological examination of infected eyes from immunocompetent mice showed sporadic foci of stromal necrosis and edema that contained fusarial conidia and hyphae in the anterior one-third of the cornea 1 day after infection. No hyphae and only occasional conidia were found in the corneal layers of immunocompetent mice 4 days after infection, with mild or no stromal necrosis. By day 8 after infection, neither fungi nor stromal edema could be found in the ocular tissue of immunocompetent mice.

In contrast, infected eyes from cyclophosphamide-treated mice revealed pronounced stromal necrosis, edema, and numerous hyphae in the anterior two-thirds of cornea 1 day after infection. The stromal necrosis, edema, and hyphae all reached Descemet’s membrane by 4 days after infection (figure 5). Hyphal invasion of the anterior chamber and lens was observed, with profuse cellular infiltration and structural destruction in one-half of cyclophosphamide-treated mice at 8 days after infection. The cellular infiltration was morphologically consistent with a neutrophilic infiltrate. Although still more severe than in immunocompetent animals, the tissue involvement was less profound in the other half of the cyclophosphamide-treated mice 8 days after infection, with hyphal invasion, cellular infiltration, and structural destruction being limited to the cornea.

In general, the hyphal invasion, inflammation with neutrophilic infiltration, and stromal destruction with marked stromal edema were most prominent in the infected corneas of cyclophosphamide-treated mice and were minimal in immunocompetent mice 4 and 8 days after infection. Mock-infected eyes were histopathologically unremarkable and appeared to be normal.

**DISCUSSION**

A reliable mammalian system is essential for the understanding of the pathogenesis of human ocular infection. Previous attempts to establish experimental keratomycosis models have used a variety of animals [14–23]. We examined the potential of developing a mouse model for *F. solani* keratitis that used corneal surface inoculation. Successful corneal surface inoculation would enable pathogenic studies of microbial adherence and the early events of fungal keratitis. Additionally, a mouse model would offer opportunities for studies of the immunology and molecular genetics of oculomycotic pathogenesis.

Developing this new model involved examining the immune status of the host and the dose of the microbial inoculum. Infection was reproducibly established in cyclophosphamide-treated mice after the application of a fungal suspension that contained conidia and hyphae to the scarified cornea. Intact corneas of healthy mice withstand fungal infection after surface inoculation and require corneal scarification [33]. Injuring the corneal epithelium by scarification is a standard method for circumventing corneal barriers [33, 36–39] and, to some degree, mimics many fungal infections of human eyes.

An absolute threshold of a fungal inoculum was not found, but as few as $1 \times 10^3$ CU of *F. solani* could lead to a mild

**Figure 3.** Clinical progression of keratomycosis in mice. Corneas of immunocompetent and cyclophosphamide-treated BALB/c mice were mock infected or infected with $1 \times 10^6$ culturable units of *Fusarium solani* and photographed 1, 4, and 8 days after infection. Representative findings are shown.
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keratitis after epithelial wounding. Increasing the number of microorganisms by 10-fold increments progressively increased the severity of disease, and an inoculum of $1 \times 10^5$ CU ensured that all corneas would initially become infected. A 5-μL volume of microorganisms was used in the effort to enhance adherence, with only a 2.5-fold reduction in the initial input titer being observed within the cornea 6 h after infection. This level of reduction is typical after topical inoculation of the eye with fungi [33] (T.G.W., K.R.W., and B.M.M., unpublished data).

Nonspecific primary host defenses, such as blinking and tearing, may contribute to the observed reduction, because part of the challenge inoculum administered is likely removed from the corneal surface by these mechanisms.

Pretreatment with cyclophosphamide, a regimen that is known to induce immunosuppression within ocular tissues [32, 33], led to increased severity and prolonged fungal persistence in the mouse cornea. Although the use of an immunosuppressive agent, such as cyclophosphamide, may be viewed to make an animal system artificial or nonrepresentative, immunosuppression allowed the development and progression of eye disease. The goals of a mouse model of keratomycosis that used an immunosuppressant are not only to mimic the extent and procession of human disease but also to create a system that is amenable to the dissection of the cellular and molecular mechanisms of fungal keratitis. Toward these goals, the severity and course of disease in this and other fungal mouse models can be experimentally modulated by adjusting the dose and strain of the fungal inoculum and by manipulating the genetic background and immunsusceptibility of the recipient host [33] (T.G.W., K.R.W., and B.M.M., unpublished data).

The present mouse model was characterized by categorizing disease severity, quantifying isolate recovery, and observing histopathological changes. The findings indicate that treatment of the mice with cyclophosphamide enhanced fungal invasion and the progression of disease. Whether cyclophosphamide enhanced fungal keratitis by affecting the immune system or by influencing nonimmune mechanisms, such as general cytotoxicity, could not be determined from our studies. The immunocompetent animals had occasional conidia in the anterior portion of the cornea, but these conidia did not produce detectable hyphae and were possibly embedded in the cornea at the time of inoculation. It is unclear whether the cyclophosphamide treatment promoted the ability of the *F. solani* to produce hyphae or the lack of the development of hyphae in immunocompetent animals contributed to the reduced virulence.

On the basis of peripheral white blood cell counts, immunosuppression with the described regimen of cyclophosphamide resulted in general systemic immunosuppression, with no significant immune reconstitution occurring even 2 weeks after treatment [32, 34]. It is important to note, however, that cyclophosphamide is cytotoxic only to immunologically active (replicating) cells and has a relatively short half-life in the body—
the plasma half-life is 4–6 h, and 50%–70% of it is secreted in the urine within 48 h [34, 40]. Nevertheless, the persistence of viable F. solani over 8 days in immunosuppressed mice suggests that corneal inflammatory reactions are involved in the pathogenesis of fungal keratitis. These findings are compatible with protective host immunity being involved in the resolution of this corneal infection. Inflammatory mediators also appear to have been responsible for part of the destructive keratitis caused by F. solani; in cyclophosphamide-treated mice, the fungal load gradually waned, whereas disease remained severe.

Histologically, infected eyes of cyclophosphamide-treated mice showed pronounced inflammatory cell infiltration throughout the corneal stroma 1 day after infection. Although corneal vascularization occurs in a similar mouse model for keratitis caused by Candida species [33], this was not significantly noticeable in the present study. Fusarial virulence factors that are known to be important in mycosis of nonocular tissues might have played a role, especially in immunocompromised mice. The fusarial mycotoxins T-2 [41, 42], deoxynivalenol [43, 44], and fumonisins [45] all have inhibitory effects on the immune system that include cytotoxicity to lymphocytes and monocytes and the reduction of neutrophil chemotaxis and macrophage phagocytic function. It is possible that the treatment with cyclophosphamide permitted the pathogenic process to begin, and fungal virulence factors then contributed to its progression.

In addition to describing a new in vivo keratomycosis model, we also report a quantifiable biological unit for Fusarium species relative to the culture optical density at 600 nm. Termed a “culturable unit,” this quantitation allowed normalization of the inocula to viability on the basis of an end-point dilution assay rather than simply a biochemical or physical attribute. Previous work with the yeast Candida albicans [33] has demonstrated that a CU is equivalent to a colony-forming unit for organisms that can be quantitated by colony formation. Of interest, the CU:OD_{600} conversion factor for Fusarium species is ~5–6 times less than that for Candida or Saccharomyces species [33, 46]. This is likely because of the higher optical density associated with the filamentous (hyphae) growth of Fusarium species, compared with the yeast conidial forms.

The studies presented here have demonstrated that human corneal fusariosis could be successfully modeled in immunosuppressed mice by use of standardized methods of inoculation and evaluation. The fusarial keratomycosis induced was inoculum-dose dependent, comparable in severity to that seen clinically in the spectrum of human disease, and affected by host factors, such as immune status, which is also evident in humans. Although human keratitis caused by F. solani can be fairly slow to become established, it can also progress rapidly and mimick keratitis caused by Pseudomonas species, which is similar to the infection profile observed in our immunosuppressed mice. Further studies of fungal genetics and fusarial virulence in ocular tissue may reveal the molecular factors responsible for fusarial invasion of the cornea and the development of fungal eye disease. Our mouse model will be useful toward this end and will offer opportunities that, because of the limitations of other models, were previously unavailable for investigating the pathogenic events in early and established Fusarium infection of the eye.

References


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