Experimental Keratomycosis in a Mouse Model

Tzu G. Wu,1 Kirk R. Wilbmmus,1 and Bradley M. Mitchell1,2

PURPOSE. To establish a murine model of corneal candidiasis that permits molecular evaluation of fungal adherence and invasion.

METHODS. Corneas of immunocompetent, methylprednisolone-treated, and cyclophosphamide-treated adult NIH Swiss and BALB/c mice were topically mock inoculated or inoculated with 10-fold increasing amounts between 100 and 100 million colony-forming units (CFU) of Candida albicans after unilateral corneal scarification. Mock-inoculated eyes served as the control. Eyes were scored daily on a 12-point scale to categorize corneal inflammation and were enucleated for quantitative fungal cultures, analysis by polymerase chain reaction (PCR), and histopathologic examination.

RESULTS. At least 100 CFU of C. albicans initiated measurable corneal infection, but 1 million or more colony-forming units were needed to induce consistent keratitis. Treatment with methylprednisolone increased disease severity in infected BALB/c mice and fungal persistence in both BALB/c and NIH Swiss mice. Treatment with cyclophosphamide increased disease severity and fungal persistence in both strains of mice. Infectious organisms were recovered by quantitative culture, and candidal DNA was detectable by PCR. C. albicans, inflammatory cells, and stromal necrosis were histologically evident within ocular tissue.

CONCLUSIONS. Although mice are innately resistant to Candida infection after corneal inoculation, moderate to severe keratomycosis can be established in immunocompromised mice by the route of corneal scarification. Although differences between mouse strains and among immunosuppressive regimens remain to be explored, this murine model provides the basis for understanding the pathogenesis of fungal infections of the cornea. (Invest Ophthalmol Vis Sci. 2003;44:210–216) DOI: 10.1167/iovs.02-0446

Fungal infections of the eye occur worldwide and are a special concern in the tropics and in situations of immunosuppression. In large series in the United States, fungal keratitis represented only 1% of corneal infections in New York City but was involved in 55% of culture-positive microbial keratitis cases in Miami, Florida. Fungal keratitis is an especially prevalent problem in India and southeast Asia. The prevalence of fungal keratitis has increased in temperate climates over the past two decades. Widespread use of corticosteroids and antibiotics is believed to be a major contributor. Ophthalmic fungi include yeasts, nonpigmented filamentous fungi, and dematiaceous filamentous fungi. With the increase in the population of immunocompromised patients, keratomycosis caused by the yeast Candida albicans is a human infection occurring with increasingly frequency. Ophthalmic candidiasis is one of the more common opportunistic infections of the eye and is capable of infecting the eyelids, cornea, and retina. In addition, C. albicans is the most common fungal contaminant encountered in eye banks and among contact lens wearers. Candida species can be found in the conjunctival flora, occurring in up to one third of otherwise healthy individuals.

Investigation of oculomycosis requires animal models that allow high reproducibility and sensitive quantitation. Although the efficacy of antifungal agents has been extensively in rabbit models, the pathogenesis of the fungal infections of the eyes remains largely unknown. Although mouse and rat models have been preliminarily examined, experimental keratomycosis is generally mild and often self-resolving in immunocompetent hosts. Depending on the fungal species, immunosuppression with corticosteroid treatment is frequently necessary to induce fungal keratitis.

The focus of this study was to develop a model of murine keratomycosis comparable to the human form of the disease. We examined the kinetics of corneal infection by C. albicans in both inbred and outbred mice, with and without immunosuppressive treatment, and found that the severity of experimental keratomycosis in mice was inoculum-dose, host, and immune-status dependent.

MATERIALS AND METHODS

Cell Cultures

The strain of C. albicans used for these studies was ATCC 32554 (American Type Culture Collection, Rockville, MD), a human isolate that has a high degree of virulence in mice when injected intravenously. The yeast was grown on Sabouraud dextrose agar (Difco, Detroit, MI) for 3 days at 25°C, harvested in sterile phosphate-buffered saline (PBS), and diluted with sterile saline to yield 102, 103, and 104 colony-forming units (CFU) per 5 μL inoculum.

 Animals

Adult female outbred NIH Swiss and inbred BALB/c mice (Harlan Sprague-Dawley, Houston, TX) were used at 6 to 8 weeks of age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice were treated with methylprednisolone (Pharmacia & Upjohn, Kalamazoo, MI) or cyclophosphamide (Sigma, St. Louis, MO) to serve as previously described immunocompromised ocular models. An intramuscular injection of methylprednisolone at 100 mg/kg body weight was administered 5 days before, 1 day before, and 1 day after corneal inoculation with C. albicans. Cyclophosphamide was administered intramuscularly at 180 mg/kg body weight 5 days, 3 days, and 1 day before the inoculation. Untreated mice were used as immunocompetent comparisons in the study.

From the 1Sid W. Richardson Ocular Microbiology Laboratory, Cullen Eye Institute, Department of Ophthalmology, and the 2Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas.

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Corresponding author: Bradley M. Mitchell, Department of Ophthalmology, NC-205, Baylor College of Medicine, 6565 Fannin, Houston, TX 77030; bmm@bcm.tmc.edu.

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The severity of keratomycosis in the animals was scored visually with the aid of a dissecting microscope and slit lamp. A grade of 0 to 4 was assigned to each of the following three criteria: area of opacity, density of opacity, and surface regularity (Table 1). A normal, unscarified mouse cornea was given a score of 0 in each category and thus had a summation score of 0. The scores from all three categories were tallied so that several as negative controls were mock inoculated with the carrier (sterile PBS). All mice were scored daily for corneal involvement. The mice were killed and the eyes enucleated at 6 hours, 1 day, 4 days, and 8 days post infection (pi) and processed for histologic examination, quantitative microbial culturing, and PCR analysis.

Clinical Scoring

The severity of keratomycosis in the animals was scored visually with the aid of a dissecting microscope and slit lamp. A grade of 0 to 4 was assigned to each of the following three criteria: area of opacity, density of opacity, and surface regularity (Table 1). A normal, unscarified mouse cornea was given a score of 0 in each category and thus had a summation score of 0. The scores from all three categories were tallied daily for each eye to yield a possible total score ranging from 0 to 12. A total score of 5 or less was categorized as mild eye disease, a total score of 6 to 9 was considered moderate, and a total score of more than 9 was severe.

Quantitative Isolate Recovery

Infected and mock-infected eyes were enucleated and individually ground in a frosted-glass grinder with 1 mL Sabouraud dextrose broth (Difco) containing 50 μg/mL chloramphenicol (Sigma). A 0.5-mL aliquot of the homogenate was centrifuged at 21,000g, and the pellet was stored at −80°C for subsequent DNA extraction and PCR analysis. A second 0.5-mL homogenate aliquot was fourfold serially diluted in Sabouraud dextrose broth containing chloramphenicol 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 endpoint.

PCR Analysis

Eye homogenate pellets were tested by molecular analysis using C. albicans–specific PCR assays previously described. Mature fungal cells were harvested from C. albicans cultures, diluted with sterile PBS to 2 × 10⁸ cells/mL, and added in the amount of 5 μL to the extraction blanks as the positive control for the extraction. Five microliters of carrier (sterile PBS) was added to extraction blanks as the negative control. The lower limit of detection for the PCR primer set was consistently at or below 10² copies of plasmid pCA1 DNA (a 4-kb EcoRI insert of C. albicans DNA) or 10³ genome equivalents using genomic C. albicans DNA as a template. A known copy number of pCA1 was used as a positive control for the PCR assays, and deionized, distilled water (ddH₂O) was used as a negative control. Thirty percent of each PCR product was resolved on a 1.8% agarose Tris-borate-EDTA gel containing ethidium bromide and visualized under ultraviolet illumination. A 100-bp molecular weight ladder (Gibco BRL, Grand Island, NY) was included on each gel for size determination of the products. PCR products of representative samples were confirmed by Southern blot analysis using a probe specific for internal sequence as previously described.

Histology

C. albicans-infected and mock-infected eyes were formalin fixed, paraffin embedded, and sectioned at a thickness of 8 μm for histologic study. The sections were deparaffinized and stained with hematoxylin and eosin (Sigma), Grocott methenamine silver (Richard-Allan Scientific, Kalamazoo, MI), and periodic acid-Schiff (Sigma).

RESULTS

Inoculation Dosage

The virulence of C. albicans was initially evaluated in immunocompetent outbred NIH Swiss mice. None of 10 mock-infected corneas showed development of visible opacity. Keratitis developed in 1 (13%) of 9 corneas infected with 10⁵ CFU, 5 (56%) of 9 corneas infected with 10⁴ CFU, 11 (85%) of 13 corneas infected with 10³ CFU, and 5 (71%) of 7 corneas infected with 10² CFU. For subsequent studies, an inoculum of 10⁴ CFU was used. Because the parameters of the infection model were optimized, a 10⁴-CFU inoculum was adequate to initiate experimental keratomycosis in 100% of the NIH Swiss and BALB/c mice, regardless of whether the animals were immunocompetent or immunosuppressed.

Visual Examination of Keratomycosis in NIH Swiss and BALB/c Mice

After the inoculation of both immunocompetent and immunosuppressed NIH Swiss and BALB/c mice with 10⁴ CFU of C. albicans, the animals were visually evaluated daily for corneal involvement for 8 days. Despite host-related variations in disease severity, the overall kinetics of the disease were similar in both types of mice (Fig. 1). All mock-infected corneas presented 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 remained unchanged. Moderate keratomycosis developed in immunocompetent mice by 24 hours pi and persisted for a week (NIH Swiss day 1 vs. day 8, P = 0.6; BALB/c day 1 vs. day 8, P = 0.6). Immunocompetent NIH Swiss mice had significantly more involved disease than immunocompetent BALB/c mice on day 1 (P < 0.001), day 2 (P = 0.01), and day 4 (P = 0.009). Methylprednisolone-treated mice developed moderate keratomycosis on day 1 that became significantly more advanced on day 8 (NIH Swiss: day 1 vs. day 8, P < 0.001; BALB/c: day 1 vs. day 8, P < 0.001). Corneal involvement of methylprednisolone-treated NIH Swiss and BALB/c mice was similar from day 1 through day 8 (P > 0.05 for all time points). Moderate keratomycosis also developed in cyclophosphamide-treated mice initially, but corneal involvement progressed to severe inflammation on day 4 and persisted through day 8 (NIH Swiss: P < 0.001 for day 1 vs. day 4, P = 0.1 for day 4 vs. day 8; BALB/c: P = 0.01).
was assigned to each of the following three criteria: area of opacity, density of opacity, and surface regularity. The scores from all three categories their corneal involvement for 8 days. Mock-infected corneas received only carrier (sterile PBS) and served as the control. A possible score of 0 to any time. As shown in Figure 3, Candida cyclophosphamide-treated mice than on day 4 (P < 0.001). Although the disease was significantly more severe in cyclophosphamide-treated BALB/c mice than cyclophosphamide-treated NIH Swiss mice on day 1 (P = 0.02) and day 2 (P = 0.004), these two groups were similar to each other after day 2 pi.

From day 1 through day 8 pi, cyclophosphamide-treated mice had more advanced corneal disease than immunocompetent (from day 1 through day 8: P < 0.001 for both NIH Swiss and BALB/c mice) and corticosteroid-treated mice (from day 1 through day 8: P ≤ 0.003 for both NIH Swiss and BALB/c mice). Corneal disease was worse in corticosteroid-treated than immunocompetent BALB/c mice at all time points (P ≤ 0.008) except on day 2 (P = 0.1). However, corneal disease in corticosteroid-treated NIH Swiss mice was similar to that in immunocompetent mice (P ≥ 0.08 from day 2 through day 8; immunocompetent mice had a higher average score on day 1, P < 0.001). Representative findings from days 4 and 8 pi are shown in Figure 2.

Quantitative Isolate Recovery of C. albicans
Corneas of immunocompetent, methylprednisolone-treated, and cyclophosphamide-treated mice were mock infected or infected with 10^6 CFU of C. albicans and analyzed by endpoint titration 6 hours, 1 day, 4 days, and 8 days after the inoculation. No C. albicans was recovered from mock-infected corneas at any time. As shown in Figure 3, Candida was cleared from the ocular tissues in both NIH Swiss and BALB/c mice with significantly fewer organisms recovered on day 8 pi from the infected corneas than on day 1 (P ≤ 0.009). Significantly fewer organisms were also recovered on day 8 pi from the corneas of cyclophosphamide-treated mice than on day 4 (P ≤ 0.001). The rates of fungal clearance in both types of mice were greater in immunocompetent mice than in their immunosuppressant-treated counterparts (immunocompetent versus methylprednisolone-treated on day 4: P < 0.001 for NIH Swiss, P = 0.002 for BALB/c; immunocompetent versus cyclophosphamide-treated on day 4: P < 0.001 for NIH Swiss and BALB/c). Most of the infected corneas were culture negative on day 4 (6/8) and day 8 (7/8) pi in immunocompetent NIH Swiss mice, while infected corneas of immunocompetent BALB/c mice remained culture positive on days 4 (7/8) and 8 (5/8) even though they had significantly reduced recovery rates (immunocompetent NIH Swiss versus BALB/c: P = 0.007 for day 4, P = 0.05 for day 8).

At 4 days pi, significantly more C. albicans was recovered from the infected corneas of both BALB/c (P < 0.001) and NIH Swiss (P = 0.041) mice treated with cyclophosphamide than with corticosteroid treatment. By 8 days pi, there was not a significant difference in the amount of Candida between the cyclophosphamide-treated and corticosteroid-treated BALB/c mice (P = 0.2), but significantly more C. albicans was recovered from the infected corneas of corticosteroid-treated than cyclophosphamide-treated NIH Swiss mice (P = 0.013). Nonetheless, visual examination suggested that NIH Swiss mice were not more adversely affected by methylprednisolone than were BALB/c mice (P = 0.2 for day 4; P = 0.07 for day 8), but BALB/c mice were more adversely affected by cyclophosphamide than NIH Swiss mice (P = 0.06 for day 4; P = 0.007 for day 8).

Molecular Analysis
To further characterize this new animal model of keratomycosis, the molecular detection and species identification of invad-
ing organisms were performed by PCR analysis using a C. albicans–specific assay. Both C. albicans–positive and –negative PCR samples were further confirmed by Southern blot analysis using a C. albicans–specific probe. As shown in Table 2, the PCR profile of the disease model correlated with quantitative isolate recovery. All mock-infected corneas were negative for C. albicans DNA. All infected corneas of immunosuppressed NIH Swiss and BALB/c mice were positive for C. albicans DNA by PCR analysis at all four time points after inoculation with 10⁶ CFU C. albicans. Whereas 12 of 12 infected corneas in immunocompetent NIH Swiss mice were positive for C. albicans DNA at 6 hours and on day 1, none (0/8) were positive on day 4 (P < 0.001), and 1 of 8 was positive on day 8 (P = 0.002). However, most infected corneas of immunocompetent BALB/c mice were positive for C. albicans DNA at all four time points (8/8 at 6 hours and on day 1; 7/8 on day 4, P = 1.0; 6/8 on day 8, P = 0.5).

**Histopathology**

Mock-infected eyes did not show signs of infection or inflammation but did acquire some microscopic epithelial changes from the initial scarification procedure. Histologic examination of infected eyes harvested 4 days pi revealed invading yeast on the corneal epithelium and in the stroma with the most significant corneal involvement observed in cyclophosphamide-treated animals (Fig. 4). Both pseudohyphae and true hyphae were observed histologically with an apparent increased relative amount of yeast in a true hyphal phase in the cyclophosphamide-treated mice. Consistent with the fungal involvement, inflammation with neutrophil infiltration and stromal destruction was most prominent in the infected corneas of cyclophosphamide-treated mice, less severe in corticosteroid-treated mice, and minimal in immunocompetent mice 4 days pi.
DISCUSSION

A reliable mammalian system is important to understanding the pathogenesis of human ocular infection. Because the cornea of naive laboratory animals resists fungal infection, developing quantifiable models of oculomycoses has been challenging. Previous attempts to establish experimental fungal keratitis have used rabbits, rats, and mice, often by intrastromal injection or corneal scarification.6,7,18–21 The mouse eye, in comparison with the rabbit cornea, offers additional opportunities for studies in the immunology and molecular genetics of the pathogenesis of oculomycosis. Using corneal surface inoculation with and without systemic immunosuppression, we examined the murine keratomycosis model by clinical, microbiologic, histopathologic, and molecular methods.

Developing a new mouse keratitis model involved, in part, examining the route and dose of the microbial inoculum. The intact corneas of healthy mice withstand infection, even when exposed to a "paste" of fungal colonies. Yet, corneal surface inoculation enables the study of microbial adherence and the early events of fungal keratitis. Injuring the corneal epithelium by scarification is a standard method for circumventing corneal barriers and may mimic, to some degree, what occurs during human infection.18 Infection was reproducibly established after applying a fungal suspension to the scarified cornea.

C. albicans was selected as the challenge microorganism because of its clinical importance, although we have been able to establish Fusarium solani keratitis and Aspergillus fumigatus keratitis, as well, in the injured mouse cornea (Wu et al., unpublished data, 2001). An absolute threshold of a fungal inoculum was not found. As few as 10^2 CFU occasionally led to keratitis after epithelial wounding. Raising the number of microorganisms by 10-fold increments progressively increased the probability of infection. An inoculum of 1 million CFU of yeast was sufficient to ensure that nearly all corneas would have initial infection. A 5-μL volume of microorganisms was used so that most of the inoculum would have an opportunity to attach. Although some microorganisms are likely to be washed away by tearing and blinking, we found only a 10-fold reduction of yeast within the cornea 6 hours after exposure.

Table 2. PCR Analysis of Mouse Eyes for C. albicans

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<th>Time</th>
<th>NIH Swiss</th>
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<tr>
<td></td>
<td>Mock*</td>
<td>Wild†</td>
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<td>(n = 14)</td>
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<tr>
<td>6 hours</td>
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<td>1 day</td>
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<td>4 days</td>
<td>0</td>
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<td>8 days</td>
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Data are the number of PCR-positive eyes. Time is time elapsed after infection.
* Mock infected.
† Immunocompetent (untreated).
‡ Methylprednisolone treated.
§ Cyclophosphamide treated.

Figure 3. Quantitative isolate recovery of C. albicans from infected corneas. Corneas of immunocompetent, methylprednisolone-treated, and cyclophosphamide-treated mice, both NIH Swiss (A) and BALB/c (B), were mock infected or infected with 10^6 CFU of C. albicans and analyzed by endpoint titration 6 hours, 1 day, 4 days, and 8 days after the inoculation. Mock-infected corneas received only carrier (sterile PBS) and served as control. The mean culture-positive endpoints (±SD) of 12 eyes per time point are plotted. The lowest points on the graph also represent the lower limit of detection of the assay.
FIGURE 4. Histopathology of keratomycosis in mice. Mouse corneas were mock infected or infected with $10^7$ CFU of \textit{C. albicans} and enucleated 4 days after the inoculation. Eyes were formalin-fixed, paraffin embedded, and sectioned at a thickness of 8 $\mu$m. The sections were deparaffinized and stained with GMS (A) or PAS (B, C). Representative findings of infected corneas from cyclophosphamide-treated BALB/c mice are shown. The micrographs include sections through the cornea (C), anterior chamber (AC), and lens (L). The dark-staining yeast were evident throughout the corneal stroma with penetration into the anterior chamber. Neutrophilic infiltration of the anterior chamber was also evident. Penetration of the lens capsule (LC) by the invading yeast (arrows) was also observed (C). Original magnification, (A) $\times$ 100; (B) $\times$ 40; (C) $\times$ 160.

Model development also involved selecting the type and immune status of the host. Interspecies differences in ocular susceptibility to corneal infection are probably important in fungal keratitis. We chose to evaluate both an outbred stock (NIH Swiss) and an inbred strain (BALB/c) of mice. Although the inbred strain would be beneficial for future immunologic and genetic studies, the NIH Swiss mice allowed us to determine the effect of genetic diversity such as that seen in the diverse human population on the infection and disease process. In an unexpected finding, we saw no greater experimental differences among the outbred animals than that observed in the inbred animals. This was true when results were compared either within a particular experiment or across independently repeated experiments. These findings are consistent with the notion that some of the factors affecting keratomycoses are independent of genetic variation. However, overall we found that inbred BALB/c mice had more severe keratitis and more fungal growth than did outbred NIH Swiss mice. Host responses could clear even a relatively high fungal inoculum within 4 days in both mouse types, but \textit{C. albicans} persisted at a higher level over 8 days in BALB/c mice. This mouse strain apparently lacks factors contributing to corneal processes that control fungal growth. Systemic immunosuppression made the two types of mice similar in susceptibility to fungal keratitis, although fungal growth still tended to be greater in immunosuppressed BALB/c mice than in immunosuppressed NIH Swiss mice.

Pretreatment with methylprednisolone or cyclophosphamide, using regimens known to induce immunosuppression in ocular tissues, led to increased severity and prolonged fungal persistence in the mouse cornea. A strain-drug interaction was detected. Methylprednisolone treatment led to the recovery of significantly more yeast after 8 days in NIH Swiss mice, whereas relatively more fungi were recoverable from cyclophosphamide-treated BALB/c mice. Whether these immunosuppressants enhanced fungal keratitis by effects on the immune system or by affecting other corneal defense mechanisms could not be determined from our studies. The presence of fungal DNA paralleled the results of quantitative isolate recovery, so most fungi persisting in the infected cornea were probably viable. Immunosuppression with cyclophosphamide in particular predisposed to intraocular fungal extension. Within 1 week after corneal inoculation of cyclophosphamide-treated mice, pseudohyphae and true hyphae penetrated Descemet's membrane, entered the anterior chamber, and invaded the anterior lens capsule.

Some investigators argue that agents such as corticosteroids make an animal system artificial or nonrepresentative, but immunosuppression is an important risk factor for human \textit{Candida} keratitis. The goals of a mouse model of keratomycosis using an immunosuppressant are not only to mimic the extent and evolution of human disease but also to create a system amenable to dissecting the cellular and molecular mechanisms of fungal keratitis. The severity and course of our murine model can be experimentally modulated by adjusting the size and strain of the fungal inoculum and by manipulating the genetic background and immunosusceptibility of the recipient host. This mouse model using topical inoculation after corneal scarification was characterized by categorizing the disease severity, quantifying the isolate recovery, analyzing the fungal DNA, and observing the histopathologic changes. The findings indicate that 1 million CFU of \textit{C. albicans} efficiently established initial infection and that immunosuppression with methylprednisolone or cyclophosphamide enhances fungal invasion and disease progression.

The persistence of viable microorganisms over 8 days in immunosuppressed mice suggests that corneal inflammatory reactions are involved in the pathogenesis of fungal keratitis. The findings are compatible with the involvement of protective cellular immunity in the resolution of \textit{C. albicans} corneal infection. Inflammatory mediators also appear responsible for part of the destructive keratitis caused by \textit{C. albicans}. In cyclophosphamide-treated mice the amount of fungi gradually waned although disease severity remained severe, occasionally leading to perforation.

Human fungal keratitis can be successfully modeled in immunosuppressed mice using standardized methods of inoculation and evaluation. This mouse keratomycosis model will be
useful for investigating the pathogenetic events in early and established fungal infection of the eye.

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References