statistical significance was due to the presence of several eyes that had high facilities, very high fixative volumes, and low B pore densities, but that nonetheless did not qualify as outliers according to the criteria established in that study. We had no such eyes in the present work and therefore observed a statistically significant correlation between B pore density and fix volume. There are several possible explanations of this data. It is possible that there is, in fact, a correlation between B pore density and fixative volume that was masked in the earlier study. Alternatively, B pore density may depend on other factors that have not yet been identified. More work is required to investigate this issue.

Some of the eyes included in this study had postmortem times greater than 20 hours, which was previously identified as a time at which changes in I pore density could be observed in human eyes. Because of the paired nature of this study, such postmortem effects would be a cause for concern if they interacted with ECA effect(s) to alter facility and/or pore statistics. We found no evidence for a postmortem interaction with ECA effects. More specifically, regression analysis of the following ratios (defined as ECA-treated eye value/contralateral eye value) against postmortem time found no statistically significant dependencies: posttreatment facility ratio ($P = 0.26$); grand facility ratio ($P = 0.35$); pore density ratio for I, B, and total pores ($P = 0.67, 0.49, 0.63$, respectively); pore size ratio for I, B, and total pores ($P = 0.82, 0.36, 0.90$, respectively); and nD product ratio for I, B, and total pores ($P = 0.83, 0.92, 0.80$, respectively). Because all statistical inferences were based on ECA/control ratios, the above results strongly suggest that postmortem time was not a confounding factor in the analysis and that the results would not have changed if all eyes included in the study had postmortem times of 20 hours or less.

### References


### Contact Lens–Induced Infection—A New Model of Candida albicans Keratitis

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**Purpose.** A model of experimental keratomycosis was established that mimics human disease in which the only fungi present are those that are actively growing within the cornea.

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**Methods.** Dutch-belted rabbits received a subconjunctival injection of triamcinolone acetonide to one eye. One day later the epithelium was removed from the central cornea and a standardized inoculum of *Candida albicans* blastoconidia was placed on the corneal surface and covered with a contact lens. The lids were closed with a lateral tarsorrhaphy. After 24 hours, the lid sutures and contact lens were removed. Five days later the animals were killed, and their corneas were subjected to separate isolate recovery and histology studies. A group of similarly infected rabbits without corticosteroid injection served as controls.

**Results.** Both groups developed invasive corneal disease. Although isolate recovery was not significantly different from corticosteroid-treated rabbits compared with controls, fungal biomass was increased. Hyphal invasion was limited to the anterior cornea in control eyes, but penetrated deep stroma in most of the corticosteroid-treated rabbits.

**Conclusions.** Invasive corneal disease can be established with a surface inoculum. Corticosteroid administration increased corneal penetration of hyphae. Quantitative isolate recovery is not a reliable measure of the fungal load within the cornea. (*Invest Ophthalmol Vis Sci. 1999;40:1607-1611.*)
Recent studies of experimental fungal keratitis have focused on inoculation of blastoconidia to establish disease. However, it has become clear to us that the residual intrastromal inoculum, the portion of inoculum that is not actively replicating but that remains viable for the course of an experiment can confound our ability to adequately evaluate experimental therapy when the measure of a drug’s efficacy is based on quantitative isolate recovery. This is not a problem with fungicidal agents, which are capable of killing Candida albicans in both quiescent and metabolically active phases. Unfortunately, most new antifungal drugs are fungistatic. This led us to develop a model in which the only fungus present in the cornea is in the invasive phase. We demonstrate the feasibility of this model with C. albicans and measure the effect of corticosteroid administration on the fungal invasion of the cornea.

**Materials and Methods**

**Inoculum**

We used C. albicans strain VE-175, a stable switch variant of strain VE-102, a human corneal isolate that we have studied previously. The inoculum was prepared by inoculating two Sabouraud’s agar plates (BBL, Coxsackie, MD) from a frozen agar plug that was preserved at −80° C. One plate was grown confluent, and the other was streaked for isolation to confirm the purity of the culture. Cultures were incubated for 24 hours at 35° C, harvested in sterile normal saline, and pelleted by centrifugation. After the supernatant was poured off, yeasts were suspended in the saline that remained in the tube, yielding a paste of yeast containing 1 × 10⁶ colony-forming units (CFU)/ml, which was pipettable using a wide-mouth pipette tip.

**Animals**

Dutch-belted rabbits of either sex weighing 1 kg to 2 kg were used in these experiments. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Corticosteroid Administration**

On the day before inoculation, rabbits that were to receive corticosteroid were given subconjunctival injections of triamcinolone acetonide (TA, 40 mg/ml, Kenalog; E. R. Squibb & Sons, Princeton, NJ) in the dosage of 1 mg (25 μl), 3 mg (75 μl), or 12 mg (300 μl). Control rabbits did not receive an injection.

**Model of Invasive Keratitis**

Dutch-belted rabbits were anesthetized with intramuscular ketamine and xylazine. Corneal anesthesia was obtained with topical 0.5% proparacaine hydrochloride. A 7-mm trephination mark was made in the central cornea of one eye, and the epithelium was completely removed from the enclosed area with a knife edge. The nictitating membrane was removed by sharp dissection. Twenty microliters of the fungal inoculum was then placed directly on the debrided area of the cornea and covered with a contact lens. A lateral tarsorrhaphy prevented extrusion of the contact lens. The animal remained anesthetized for at least 2 hours after inoculation.

### Table 1. Measures of Fungal Recovery from Rabbits Infected with Candida albicans

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isolate Recovery (log CFU)</th>
<th>Fungal Biomass (log₂ (area in μm²))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.32 ± 0.75</td>
<td>12.40 ± 0.87</td>
</tr>
<tr>
<td>1 mg TA</td>
<td>13.90 ± 1.14</td>
<td>13.84 ± 1.33</td>
</tr>
<tr>
<td>3 mg TA</td>
<td>16.24 ± 1.14</td>
<td>13.78 ± 1.33</td>
</tr>
<tr>
<td>12 mg TA</td>
<td>13.52 ± 0.99</td>
<td>16.68 ± 1.15</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n is number of rabbits.

The tarsorrhaphy sutures and contact lens were removed 24 hours later. In the first experiment one group of animals served as controls, and the other received 12 mg subconjunctival TA. The second experiment consisted of three groups of three animals each: group one received 1 mg TA, group two received 3 mg TA, and group three was a control group.

**Postmortem Tissue Dissection**

Rabbits were killed by rapid intravenous injection of euthanasia solution. The infected corneas were removed at the limbus and bisected longitudinally through the middle of the infected area. One half of the cornea 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 and processed for quantitative isolate recovery using our standard method of homogenizing the cornea and plating samples of serial dilutions in triplicate on Sabouraud's agar.

**Histology**

For each cornea, three 5-μm, paraffin-embedded sections were cut at 20 intervals of 100 μm from the midcornea. The sections were stained by the Gomorri’s methenamine silver method to visualize fungi. Infected corneas were stained by the Gomorri’s methenamine silver method to visualize fungi. Infected corneas were stained by the Gomorri’s methenamine silver method to visualize fungi. Infected corneas were stained by the Gomorri’s methenamine silver method to visualize fungi. Infected corneas were stained by the Gomorri’s methenamine silver method to visualize fungi. Infected corneas were stained by the Gomorri’s methenamine silver method to visualize fungi. Secondly, we used an image analysis system that was coupled to a computer with an Intel Pentium® processor. This allowed us to measure total surface area of the fungal elements contained in each corneal section (fungal biomass). By adjusting the lighting for each section scanned, we were able to capture an image from the micro-mam that was sufficient to differentiate the hyphal elements from the background tissue, based on gray scale density. The settings within the software were then adjusted to “tag” the fungal elements according to their level of relative “darkness” compared with the background. Automation of this process was not possible because of confounding staining by inflammatory cells. However, by direct comparison of the gray scale image to the slide on the microscope, we were able without difficulty to delete manually any erroneously “tagged” objects. Once all the hyphae were tagged, the computer determined the area. One section at each 100 μm interval was scanned microscopically, and the total cross-sectional area of the hyphal elements was measured in squared-micrometers.
Statistical Analysis

We pooled the data from both experiments for analysis because there were no significant differences in the data from the control groups in the two experiments. Because the effect of treatment was modeled to be proportional to the pretreatment level of disease, a log transformation of the data was done. Standard analysis of variance methods were used to examine the effect of corticosteroid administration on disease. A further analysis of the biomass data was done to determine the reliability of using only one, two, or three corneal sections to determine the total fungal biomass compared with multiple sections cut through the entire diameter of the fungal lesion. The correlation between isolate recovery and fungal biomass also was examined to determine whether these two measures of response were different. All statistical analyses were performed using SAS for Windows (SAS Institute, Cary, NC).

RESULTS

All rabbits in both experiments became infected, with sufficient disease in control rabbits to permit histologic measure-
ment of hyphal invasion into the corneal stroma (Table 1). Although isolate recovery was consistently higher from rabbits treated with corticosteroid compared to control rabbits, this trend did not achieve statistical significance. In control corneas, hyphal invasion was limited to the anterior stroma and generally was sparse (Fig. 1). In rabbits treated with corticosteroid, fungal biomass increased in all corneas, manifested by an increase in the density of fungal elements and to a lesser extent by an increased depth of penetration (Fig. 2). Fungal biomass increased progressively with increasing dose of corticosteroid administered. There was a statistically significant difference for fungal biomass measurements only between the group of rabbits that received the highest dose (12 mg) of corticosteroid per day and control groups (P = 0.01).

To examine the reliability of determining fungal biomass by evaluating fewer corneal sections, we first considered control rabbits. In these eyes, an R value of 0.99 was obtained when the data from one corneal section of each eye was compared with the entire data set of 20 corneal sections. When all groups of rabbits were considered (treated and untreated controls) the correlation fell slightly (R = 0.92). When the data from three sections for each eye were pooled and compared with the entire data set, the correlation for control rabbits was 0.98 and for all rabbits groups it was 0.99.

To confirm that the fungal biomass was, in fact, a different measure of response to therapy than isolate recovery in this model, we examined the correlation between isolate recovery and fungal biomass data. For the group of untreated control rabbits, the correlation was poor (R = 0.39). If all rabbits were considered, the value for R dropped to 0.25.

**DISCUSSION**

The development of a reliable animal model of fungal keratitis has been an elusive goal for researchers in ocular fungal disease. Normal laboratory animals, even more so than humans, appear resistant to the most common fungal pathogens so that the reliable establishment of infection remains a fundamental problem. In our laboratory we have developed several models of fungal keratitis for use in the study of pharmacological agents, but these models, while useful experimentally, appear inappropriate for the study of certain classes of agents such as fungistatic drugs. Moreover, they do not permit the study of events in the initiation and development of corneal infection.

The complex life cycle of fungi is an obstacle to the development of precise methods for quantifying the disease, an essential element in any model used in therapeutic research.

With bacteria, it is relatively straightforward to estimate organism content using colony-counting techniques. For fungi, the fact that every nucleus-containing hyphal fragment has the potential to form a colony introduces a large degree of uncertainty in organism counts. In addition, when the inoculum (composed of blastoconidia) is deposited in the corneal stroma, there is evidence that nonproliferating blastoconidia remain viable for a long time (DM O’Day, unpublished data, 1998), although hyphal proliferation may not occur. These surviving, but not replicating, colony-forming units contribute to a background colony count that limits the detection of small, but significant changes in fungal populations.

In setting out to establish a valid model of fungal keratitis, we recognized four essential components: (1) the method of initiation of infection should approximate the way human infection occurs; (2) immunologic manipulation of the host animal should be unnecessary; (3) the invasive fungal elements should be exclusively in the hyphal phase; and (4) quantification of disease should be objectively measurable. Previous studies have shown Dutch-belted rabbits to be suitable experimental animals for these studies. For the inoculum, we chose C. albicans strain VE-175 on the basis of our previous studies with this organism. The method of inoculation was developed on the analogy of human infection after contact lens wear. Excision of the nictitating membrane and a temporary tarsorrhaphy were performed to enhance lodgment of the inoculum on the bare stromal surface so that adequate time for invasion could occur before the nonattacked organisms were swept from the corneal surface. In this way, we hoped to have only hyphal-phase organisms in the corneal stroma.

The previous difficulty in objectively measuring this disease led us to attempt quantitative measurement of the amount of fungus (fungal biomass) in corneal sections. These data strongly support both the feasibility and practicality of this approach.

This model appears to mimic human corneal infection: blastoconidia deposited on the debrided corneal surface germinate and produce hyphae, which invade the corneal stroma. Despite the brief 5-day period of observation, histologic sections reveal a relatively uniform stromal invasion by healthy hyphae. From two perspectives these are important observations: they indicate that the bare nontraumatized corneal stroma is susceptible to fungal infection exclusively in the hyphal phase by this strain of C. albicans and that tissue invasion can be evaluated histologically in this model without the need to examine large numbers of serial sections.

The second part of the study demonstrated the utility of the model by confirming previous reports of the effect of corticosteroid on fungal invasion. Again, fungal growth was exclusively in the hyphal phase. As we anticipated, colony counts failed to correlate with the estimated fungal biomass; however, this does highlight a problem—the lack of a gold standard for measuring fungal growth in tissue. Clearly, although this quantitative histologic scanning technique will provide a robust measure of fungal growth, because the baseline is zero, assessing viability remains an open question that cannot be resolved by population estimates based on colony counting techniques. The development of sensitive quantitative measures of fungal growth in tissue is an essential element for future progress. Techniques that can measure events at this molecular level may be the key and are the focus of our ongoing research.

**References**


Immunopathology of Pineal Glands from Horses with Uveitis

Carolyn M. Kalsow,1 Richard R. Dubielzig,2 and Ann E. Dwyer1,3

PURPOSE. Pinealitis accompanying uveitis is well established in laboratory models of experimental autoimmune uveoretinitis. In naturally occurring uveitis, pinealitis has been demonstrated in the pineal gland from a mare with active uveitis and is suspected in some human uveitides. We have evaluated pineal glands from horses with various stages of uveitis for signs of immunopathology accompanying spontaneous uveitis.

METHODS. Pineal glands from 10 horses with uveitis and from 13 horses without uveitis were evaluated for histoch- emical (H&E, collagen) and immunohistochemical (MHC class II antigen expression, infiltration of T and B lymphocytes, and glial fibrillary acidic protein (GFAP) and vimentin upregulation) evidence of inflammation.

RESULTS. Septal areas of pineal glands from horses with uveitis had clusters of MHC class II antigen-expressing cells, T lymphocytes, and enhanced collagen deposition. These changes were not as readily observed in pineal glands from horses without uveitis. B lymphocytes were detected only in the pineal gland from the one mare with active uveitis in which T and B lymphocytes were organized into follicles. No differences in GFAP or vimentin immunoreactivity were noted in pineal glands from horses with or without uveitis.

CONCLUSIONS. These pineal gland changes suggest that the pinealitis associated with equine uveitis is transient just as the uveitis of these horses is recurrent. Study of pineal glands from horses with clinically documented uveitis allows demonstration of subtle pineal changes associated with natural uveitis. Similar changes would be difficult to document in human patient populations.

Lymphocytic infiltration of the pineal gland is regularly observed in laboratory models of experimental autoimmune uveoretinitis (EAU). This experimental autoimmune pinealitis (EAP) is a generalized phenomenon that has been induced in a variety of species by various photoreception-associated proteins that are also present in pineal gland.1-4

In humans, lower serum levels of the pineal neurohor- mone melatonin5-7 in patients with uveitis suggests pineal gland abnormalities in these individuals. However, there is no direct histopathologic evidence of pineal gland changes in human patients with uveitis. Affected pineal glands are usually not available for direct observation, especially at the time of active inflammation, and noninvasive techniques have yet to be developed to detect pineal inflammation in situ.

Equine recurrent uveitis is a natural disorder that can serve as a model of human uveitis.8 This is a spontaneous inflammation in which there is no artificial perturbation of the systemic immune response, and tissues from horses euthanatized because of blindness or other reasons are more readily obtainable for study than are tissues from human patients. Documented pinealitis in a mare with active uveitis,9 prompted this investigation of immunohistopathologic changes of pineal glands from several horses with and without uveitis.

MATERIALS AND METHODS

Pineal Glands

Pineal glands from 10 horses with a diagnosis of uveitis10,11 and from 13 horses without uveitis were provided by Ann Dwyer of the Genesee Valley Equine Clinic, William Rebhun, DVM, of Cornell University, and Richard Dubielzig, DVM, of the University of Wisconsin. The tissue was recovered from horses at the time of euthanasia or natural death, fixed in formalin, 95% ethanol or Bouin’s solution and embedded in paraffin.

Clinical information was available for 6 of the 10 horses with uveitis. All 6 had had a recurrence in one eye within one year and had a duration of uveitis for greater than a year at the time of euthanasia. Only one horse was considered to have active uveitis at the time of euthanasia, i.e., recurrence within 2 weeks rather than 2 months or greater. These horses had been treated expeditiously.11 The other 4 horses with uveitis...