Efficacy of Antifungal Agents in the Cornea

I. A Comparative Study

Denis M. O'Day, Richard Robinson, and W. Steven Head

A standardized model of *Candida albicans* keratitis was developed in pigmented rabbits using a quantitative mycologic technique to evaluate the disease at intervals throughout the course. In this model, using two different infecting strains, the efficacy of five antifungal agents was compared. Amphotericin B, in concentrations of 0.5% to 0.075%, was superior to all other agents tested. Nystatin 5% ranked next, followed by 1% flucytosine, and 1% miconazole. Ketoconazole 1% was ineffective. Invest Ophthalmol Vis Sci 24:1098-1102, 1983

The selection of appropriate therapy for fungal corneal infection remains an unsettled question. Although there is considerable clinical evidence to support the use of certain agents,†-§ objective experimental data is still lacking because of the difficulties associated with the development of a suitable animal model of fungal disease. In this paper we describe a model of *Candida albicans* infection in the rabbit eye, using quantitative mycologic techniques, and its application to a comparison of efficacy of a variety of antifungal agents.

Materials and Methods

Development of the Animal Model

**Inoculum:** Two-day-old cultures of *C. albicans* grown on trypticase soy agar with 5% sheep blood (BBL) were used to inoculate the rabbit cornea. A suspension of the inoculum in normal saline in a concentration of $5 \times 10^9$ per ml was prepared and stored at 4°C over night. In preliminary studies only two of the five strains tested produced significant disease and these were used in subsequent studies (Strain LV and Strain F357). The susceptibility of these two strains to five antifungal agents, using a modification (personal communication, Dan B. Jones, 1980) of the method of Shadomy, was shown in Table 1. In vitro, the LV strain is susceptible to flucytosine at a concentration of 0.30 μg/ml but resistant to ketoconazole (minimal inhibitory concentration [MIC] > 50 μg/ml), whereas strain F357 is resistant to flucytosine (MIC > 50 μg/ml) but susceptible to ketoconazole (MIC = 0.10 μg/ml). The discrepancy between the inhibitory and fungicidal values with these agents is indicative of a fungistatic rather than a fungicidal mechanism of action. In contrast, the levels with amphotericin B suggest a fungicidal mechanism in vitro.

**Animals:** Pigmented outbred male rabbits, 1.5-3 kg were used in all experiments.

**Infection protocol:** Rabbits were anesthetised with either intravenous pentobarbital (Nembutal) or intramuscular Ketamine and xylazine hydrochloride. A retrobulbar injection of 1% xylocaine was given and topical anesthesia was achieved with Alcaine 0.5%. Using an operating microscope, eight wells, one-half stromal thickness in depth, were trephined in the cornea of each eye in a regular pattern. A glass trephine prepared from a microhematocrit tube 1 mm in diameter was used. A second trephine loaded with *C. albicans* suspension was used to inoculate each well. The trephine was placed in each well, rotated three to four times and then removed. Each well was inoculated a second time with a new trephine loaded with fresh inoculum. If inadvertent corneal perforation occurred the animal was removed from the study.

**Isolate recovery:** At predetermined intervals the animals were sacrificed with a commercially prepared euthanasia solution T-61 (Taylor Pharmacal, Decatur, IL). The whole cornea was removed by excision at the limbus, placed in a sterile petri dish, and cut into small pieces. These were then ground in a tissue grinder (Ultraturex Model SOT) for three 10-sec intervals in 3 ml of sterile saline. Ten and 100 μl aliquots of this corneal suspension were plated in triplicate on trypticase soy agar with 5% sheep blood.
Table 1. In vitro susceptibility (µg/ml)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amphotericin B</th>
<th>Natamycin</th>
<th>Ketoconazole</th>
<th>Flucytosine</th>
<th>Miconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
</tr>
<tr>
<td>LV</td>
<td>0.2</td>
<td>0.39</td>
<td>6.25</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>F357</td>
<td>0.1</td>
<td>1.56</td>
<td>3.12</td>
<td>25</td>
<td>0.10</td>
</tr>
</tbody>
</table>

MIC = minimal inhibitory concentration. MFC = minimal fungicidal concentration.

After 48 hrs incubation at 25 C the colony forming units (CFU) were counted, and the number of CFU per whole cornea was calculated based on a 3-ml total volume.

Evaluation of Topical Antifungal Agents in the Model

Agents evaluated: Polyenes: Amphotericin B 0.5%, 0.15%, and 0.075% in distilled water (Fungizone®, E. R. Squibb & Son, Princeton, NJ) and Natamycin 5% suspension (Alcon Laboratories, Fort Worth, TX). Imidazoles: Miconazole 1% in Cremophor (Janssen Pharmaceuticals, New Brunswick, NJ) and Ketoconazole 1% in polyethylene glycol 400 (Janssen). Pyrimidine: Flucytosine 1% solution in normal saline (Roche Laboratories, Nutley, NJ).

Treatment protocol: Treatment was begun 30 min after inoculation. Animals were assigned randomly to a treated or untreated control group. Six rabbit eyes were exposed to each treatment regimen with both eyes of each animal receiving either the test drug or no treatment. Drops were administered 10 times per day at hourly intervals. Eighteen hours after the conclusion of the treatment period both treated and control animals were killed and the corneas processed.

The following experiments were performed:

a. Candida albicans strain LV: a comparison of all five agents (natamycin 5%, amphotericin B 0.5%, miconazole 1%, ketoconazole 1% and flucytosine 1%).

b. Candida albicans strain LV: a comparison of different dilutions of amphotericin B in distilled water, 0.5%, 0.15%, 0.075%.

c. Candida albicans strain F357: a comparison of 1% flucytosine, 1% ketoconazole and 0.15% amphotericin B.

Results

Animal Model

Within 24 hours of inoculation a ring of infiltrate developed around the base of each trephination. This area of infiltrate grew larger over the next 36 hrs and then slowly diminished as the infection resolved clinically over a 5-day period. With both strains the course of the disease was similar and paralleled the quantitative isolate recovery rate at the end of each 24-hr period. During the first 48 hrs the isolate recovery rate was high (Fig. 1), but by 72 hrs it had fallen to 10% of the 24-hr value and by the fifth day it was less than 1%.

Fig. 1. The normal course of disease following inoculation with two different strains of Candida albicans as measured by the number of CFU/cornea. Each data point represents six eyes (mean and range).
Although the range at each sampling point was quite broad, the course of the disease was consistent with each isolate. Because the most active infection in the untreated model occurred in the first 48 hrs, evaluation of the response to drug therapy was restricted to this period.

Drug Studies

Due to a multiplicative error structure, the response variable analyzed was \( \log_e (1 + \text{CFU}) \). Preliminary analysis showed a highly significant correlation between right and left eye results \((P < 0.0001)\) in both treated and untreated groups. Hence, a nested analysis of variance was used to test differences between treatments. The error term was between rabbit mean squares.9

In the experiment comparing the efficacy of five antifungal agents against the LV strain, there was a marked variation in the response to treatment (Figs. 2, 3). The most effective drugs as a class were the polyenes, 0.5% amphotericin B and 5% natamycin, but only amphotericin B was able to eradicate infection in 48 hours. Both 1% miconazole and 1% flucytosine significantly reduced the number of CFU/cornea at 24 and 48 hrs but were far inferior to the polyenes. Treatment with 1% ketoconazole was ineffective with this strain.

Based on the excellent response to 0.5% amphotericin B, a further study was performed with concentrations of 0.15% and 0.075%. As can be seen in Figure 2, these were also highly effective. After 48 hours of treatment infection was eliminated in most eyes.

The final experiment was designed to study the relationship between in vitro derived susceptibility data and the in vivo therapeutic response. The response to treatment of strain F357 (resistant to in vitro flucytosine but susceptible to ketoconazole) was compared to that of the LV strain (resistant to ketoconazole, susceptible to flucytosine). Treatment with amphotericin B was used as a positive control (Fig. 4). Ketoconazole was no more effective against
**Candida albicans - Strain F357**

![Graph](image)

**Discussion**

The principal topical antifungal agents currently available for the treatment of corneal disease have been derived, with the exception of natamycin, from systemically administered compounds. They are used in concentrations that are empirical and appear limited only by toxicity and solubility. Over the years there have been numerous attempts to examine the efficacy of these antifungal agents in the external eye but these efforts have been largely frustrated by the lack of good models of disease, as well as a means of quantifying the response. As a result, we continue to rely on accumulated data derived largely from uncontrolled clinical studies.

The development of appropriate models of fungal corneal disease that incorporate an accurate measure of the response would offer an opportunity to examine therapy of fungal corneal disease in a way not hitherto possible. Such models should parallel human disease in terms of infecting organisms and clinical manifestations and should not require alteration of the host response. The disease process should be of sufficient duration to permit therapeutic manipulation but not of a degree of severity that would defeat therapeutic intervention. Finally, an objective method of evaluating response to therapy must be used. The application of quantitative techniques to mycologic studies in the cornea offers a more precise way to achieve objective and comparable data. In the present model, using these techniques we have defined the natural course of the disease with two separate isolates. With each, the infection tends to resolve over a period of 5 days, but during the first 48 hrs there is sufficient disease to allow therapeutic manipulation. The fact that only two of five isolates produced adequate disease emphasizes the difficulties associated with this type of experimental infection.

For the efficacy studies, we selected five agents that have been advocated for the treatment of Candida infections using currently recommended concentrations, the purpose being to determine the relative efficacy of the agents. The clear superiority of the polyenes in this external ocular model is remarkable (Figs. 2, 3). Flucytosine, the next most effective agent, produced a smaller but significant reduction in CFU. Most disappointing was the inferior efficacy of miconazole and the lack of any discernible therapeutic effect with ketoconazole despite its promise as a systemic therapeutic agent for Candida infection. While it must be emphasized that an experiment that demonstrates a cure of a fungal corneal infection within two days does not parallel human experience, the fact that under the same conditions all other agents failed to match this performance must be considered of some significance.

Since the LV strain showed in vitro resistance to ketoconazole, it could be argued that ketoconazole was ineffective for this reason. The strain was also resistant to miconazole. The availability of another strain (F357) susceptible in vitro to ketoconazole but resistant to flucytosine afforded an opportunity to compare in vitro and in vivo antifungal activity for these two drugs. Our animal studies demonstrated a
lack of correlation with the in vitro data for ketoconazole, with no therapeutic response observable against either strain. However, with flucytosine the in vitro susceptibility tests appeared to predict to some degree the in vivo response although it was still markedly inferior to either of the polyenes. There are obviously many reasons why a topical drug may fail to act, and this study has not examined other factors that may affect bioavailability. Such studies are needed before the relationship between in vitro and in vivo data can be fully evaluated.

Although treatment with 0.5% amphotericin B eradicated the infection in 48 hrs, animal and human experience would indicate that at this concentration toxicity can still be a problem with prolonged use. For this reason, we studied the efficacy of the drug at reduced concentrations. Even at a concentration of 0.075%, amphotericin B given topically is superior of 0.075%, amphotericin B given topically is superior to natamycin, the next most effective drug. In view of the overwhelming superiority of dilute amphoter-

INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE / August 1983

References

15. Newmark E, Ellison AC, and Kaufman HE: Combined pi-
17. Stern GA, Okumoto M, and Smolin G: Combined ampho-
18. Heil RG: Systemic Candidosis and Candidemia. In Ketocon-
azole in the Management of Fungal Disease, Levine HB, edit.
Sydney, Australia, Adis Press, 1982.

Key words: Candida albicans, fungal corneal infection, antifungal agents, amphotericin B, natamycin, miconazole, ketoconazole, flucytosine