In Vitro and In Vivo Susceptibility of Candida Keratitis to Topical Polyenes

Denis M. O'Day,* Wayne A. Ray,† Richard D. Robinson,* W. Steven Head,* and Alice M. Savage‡

The susceptibility of Candida albicans to topical amphotericin B and natamycin was evaluated in a model of stromal keratitis in Dutch-belted rabbits and compared with minimal inhibitory concentrations in vitro. Treatment was delayed 24 hr to allow invasive disease to occur and was then continued for 5 days. Ten strains of Candida albicans comprised the test panel. For amphotericin B, the minimal inhibitory concentration (MIC) by tube dilution classified the same strains as resistant or susceptible as did the in vivo response. A dose–response was observed with different concentrations of the drug. For natamycin, the MIC misclassified two strains. The rate of administration of natamycin required in this model was much higher than for amphotericin B, a therapeutic effect being observed with natamycin only when the drug was administered every 30 min during the in vivo efficacy and in vitro susceptibility with these strains is in agreement with that observed in the authors’ previous studies using a model of immediate treatment. Invest Ophthalmol Vis Sci 28:874–880, 1987

The relevance of in vitro susceptibility testing to in vivo efficacy for antifungal agents remains an unsettled question.1–3 In a recent study, the authors used a model of Candida keratitis in rabbits to examine this relationship for two polyenes, amphotericin B and natamycin, with a panel of C. albicans strains.4 A good correlation was established between minimal inhibitory concentrations (MIC) estimates by the tube dilution method and the efficacy of both drugs when administered topically.

Although this is the first time such a relationship has been documented, there are certain aspects of the model the authors used that raise questions about its similarity to human disease, including the superficial location of the infection, the 48-hr period of treatment, and the time treatment begins. In this model, the treatment period starts 1 hr after inoculation and it could be argued that disease is unlikely to be established at this stage because the yeast is still largely in the blastospore phase. With these concerns in mind, the authors decided to test the accuracy of their findings in another model of infection more akin to human disease. They initiated infection in the corneal stroma of Dutch-belted rabbits and delayed treatment 24 hr to allow transformation to the hyphal phase and tissue invasion to occur. In contrast to the previous experiment, treatment is continued for 5 days. Finally, the authors evaluated several regimens of differing intensity.

The authors’ objectives were to determine whether the classification of the studied strains, as established in vitro and in the previous model, held in this new model and whether a dose–related response could be established.

Materials and Methods

Isolates

Ten isolates of C. albicans, all derived from human disease, were used in the study. Each was obtained from a different patient. They were selected from a larger panel of strains that have been shown in previous studies to produce a consistent level of disease in the cornea of Dutch-belted rabbits.4 Five of these were classified as resistant to amphotericin B on the basis of the MIC and the response in the previous model of immediate treatment, and five were classified as susceptible. The isolates were prepared for study as in our previous experiments.4,5

Agents Evaluated

Amphotericin B 0.15% and 0.075% drops were formulated from Fungizone® purchased from the Vanderbilt Hospital pharmacy. A fresh vial of Fungizone® (0.5% amphotericin B) was prepared at the beginning of each experiment and refrigerated. Each morning the amphotericin B was diluted in distilled water to a final concentration of 0.15% or 0.075% for use that day. Natamycin was prepared in advance as a 5% suspension from natamycin powder (Alcon Laboratories, Fort...
Worth, TX). The formulation was identical to the commercial preparation. Drops were refrigerated until needed.

In Vivo Efficacy Studies

For these studies, C. albicans infection was established in the corneal stroma of Dutch-belted rabbits.

To prepare an inoculum, an agar plug of the isolate to be studied was removed from the freezer, placed on a plate of Sabouraud's dextrose agar and incubated for 24 hr at 32°C. Blastospores were harvested and resuspended in normal saline at a concentration of $5 \times 10^9$ colony-forming units (CFU)/ml.

Inoculation Technique

Rabbits were anesthetized with intramuscular Ketamine hydrochloride and Xylazine hydrochloride. Topical anesthesia was achieved with Opthaine 0.5%. Using the operating microscope for visualization, a 30-gauge needle attached to a 250 µl Hamilton gas-tight syringe was introduced into the corneal stroma 2 mm from the limbus and advanced to the central cornea. Twenty-five microliters of the spore suspension were injected, and the needle was removed. If penetration of the anterior chamber occurred, the animal was removed from the study.

This technique has been used in previous studies but, in an important departure from our previous work, treatment is delayed 24 hr to allow the infection to become established.

Treatment

For all experiments in this study treatment was begun 24 hr after inoculation. In each group except the untreated controls, the corneal epithelium was removed each day by first marking a 7-mm disc with a disposable trephine and then gently removing the epithelium with a number 15 Bard-Parker blade (Bard Parker Co.; Rutherford, NJ). In the experiments involving amphotericin B, both eyes of each animal received the same treatment or no treatment. When natamycin was to be administered, only one eye from each animal was infected because the discomfort caused by frequent natamycin application was judged to be too severe if given to both eyes. This animal utilization conforms with the ARVO Resolution on the Use of Animals in Research.

Animals were assigned at random to an untreated control group and various treatment groups. For amphotericin B experiments, animals were treated with amphotericin B 0.15% or 0.075% 10 times a day. Our standard method of instilling the antifungal preparation was followed. In preliminary experiments, it was established that a more frequent rate of administration was unnecessary for amphotericin B.

For natamycin, groups were treated with the 5% suspension every 30 min for 24 hr or every hr for 24 hr. Treatment was continued for 5 days. Preliminary experiments established that when treatment was delayed 24 hr, neither the administration of one drop per hour for 10 hr, or one drop per 30 min for 10 hr produced a therapeutic effect with strains shown to be susceptible in previous studies when the same treatment was initiated 1 hr after inoculation.

Eighteen hours after the last drops were instilled, all the animals were killed and the number of CFU recovered from each cornea was estimated using a standardized isolate recovery technique. Because the effect of treatment is modeled to be proportional to the pretreatment level of disease, each CFU was expressed as a logarithm to the base 2 (LCFU). When two eyes were used, as in the amphotericin B experiments, the log2 CFU values for the two eyes were averaged (ALCFU). This single value for each animal was the appropriate unit of analysis because the two single eye measurements within an animal were correlated.

The effect of a drug treatment was estimated by subtracting the mean of the LCFU or ALCFU measurements of the treated from the control animals, yielding a difference termed $\Delta$. A $\Delta$ of 0 implies no response to treatment; large $\Delta$ values represent good responses to treatment and small values represent a poor response. An estimate of the standard error of $\Delta$ was calculated using analysis of variance techniques.

Evaluation of In Vitro Susceptibility

All the strains in this experiment were evaluated previously for in vitro susceptibility to amphotericin B and natamycin by three different methods. Our previous studies showed that tube dilution MIC values correlated best with in vivo susceptibility. These MIC data from the previous experiment, comprising the average of three individual determinations obtained in separate experiments, were used in this study.

Results

Histology studies at 24 hr after inoculation showed the organism to be invading the corneal stroma actively. Although blastospores are still present, pseudohyphae and true septate hyphae are developing and invading adjacent corneal stroma (Fig. 1). Seven days after inoculation, the level of disease as measured by isolate recovery rates in the control animals for all 10 isolates in the amphotericin B studies, and 9 isolates in the natamycin experiments ranged from 15.3–20.2 LCFU per cornea (Tables 1, 2).
Amphotericin B

All five strains of Candida albicans determined to be susceptible to amphotericin B in vitro (Table 1, d, g, h, k, l) and in vivo in the model of immediate treatment were found to be susceptible to the drug. With the 0.15% preparation, Δ LCFU ranged from 4.9–12.1 (P < 0.01). A similar effect was noted with the 0.075% concentration (Table I).

A dose–response relationship was observed when a pooled analysis was performed for all five susceptible strains. The average difference in Δ LCFU between the 0.15% and 0.075% amphotericin B treatment regimens was 2.1 log₂ CFU (P < 0.01).

None of the strains classified as resistant to amphotericin B in our previous studies (Table 1, a, b, c, j, q) were susceptible in this model when either concentration was used (Δ 0.15% [0.6–1.5], Δ 0.075% [0.3–1.13] P, not significant). Thus, the classification of strains as susceptible and resistant was completely concordant with the classification using the MIC by tube dilution method and with the classification from the previous in vivo model. Overall, 85% of the variance in in vivo response was explained by the in vitro MICs (P < 0.001, Fig. 2). There was also good agreement between the response of these strains in the two in vivo models (R² = 0.8, Fig. 3). We noted that response to amphotericin B in the model of delayed treatment more closely approximated the dichotomous response expected on the basis of the MIC than did response in the immediate treatment model.

Natamycin

Agreement with response in immediate treatment model: There was good agreement between the re-

Table 1. Amphotericin B: susceptibility of 10 isolates of Candida albicans as measured in vitro and in models of immediate and delayed treatment

<table>
<thead>
<tr>
<th>ID</th>
<th>Class</th>
<th>MIC (μg/ml)</th>
<th>Immediate treatment* (ALCFU)</th>
<th>Delayed treatment (ALCFU)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>AmB 0.15% (Δ)</td>
<td>Control</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AmB 0.15% (Δ)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AmB 0.075% (Δ)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SE</td>
</tr>
<tr>
<td>d</td>
<td>S</td>
<td>0.05</td>
<td>16.70</td>
<td>19.00</td>
</tr>
<tr>
<td>g</td>
<td>S</td>
<td>0.05</td>
<td>19.90</td>
<td>16.40</td>
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<tr>
<td>h</td>
<td>S</td>
<td>0.04</td>
<td>16.80</td>
<td>18.90</td>
</tr>
<tr>
<td>k</td>
<td>S</td>
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<td>17.80</td>
<td>16.60</td>
</tr>
<tr>
<td>l</td>
<td>S</td>
<td>0.05</td>
<td>17.00</td>
<td>18.50</td>
</tr>
<tr>
<td>a</td>
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<tr>
<td>b</td>
<td>R</td>
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<td>16.20</td>
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<tr>
<td>c</td>
<td>R</td>
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<td>R</td>
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<td>q</td>
<td>R</td>
<td>0.42</td>
<td>17.80</td>
<td>19.30</td>
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</tbody>
</table>

* Data previously reported.
† ALCFU = average of the two eyes log₂ (CFU) reading for each animal in which CFU is the estimated number of colony-forming units per cornea.
‡ ID = identification number of each strain.
§§ = susceptible and R = resistant; based on in vitro MIC testing.
Δ = the difference between mean ALCFU for the control animals and mean ALCFU for the treated animals.
Table 2. Natamycin: susceptibility of nine isolates of *Candida albicans* as measured in vitro and in models of immediate and delayed treatment

<table>
<thead>
<tr>
<th>ID</th>
<th>Class§</th>
<th>MIC (µg/ml)</th>
<th>Control</th>
<th>Nat (A)</th>
<th>Delayed treatment model (ALCFU)†</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
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<tr>
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<td>S</td>
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<tr>
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<tr>
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<tr>
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<td>S</td>
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<td>17.00</td>
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<tr>
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</tr>
<tr>
<td>j</td>
<td>R</td>
<td>33.30</td>
<td>16.20</td>
<td>0.80</td>
<td>19.60</td>
</tr>
</tbody>
</table>

* Data previously reported.4
† ALCFU = average of the two eyes log₂ (CFU) reading for each animal in which CFU is the estimated number of colony-forming units per cornea.
‡ ID = identification number of each strain.
§ S = susceptible and R = resistant; based on in vitro MIC testing.

- **Response of these strains in the two in vivo models (R² = 0.78)** (Fig. 4). Five of the six strains classified as susceptible to natamycin on the basis of response in the immediate treatment model were also susceptible in this model when treatment was delayed 24 hr and then administered every 30 min for 5 days (Δ, 2.4–5.50) (Table 2, Fig. 4). One strain (g) shown to be susceptible to immediate treatment did not respond to this delayed regimen. The three strains resistant in our previous in vivo studies also failed to respond. All nine strains were resistant to natamycin given at the reduced rate of 1 drop/hr.

- **Agreement with MIC:** Three of four strains classified as resistant in vitro to natamycin (MIC, 3.10–5.20 µg/ml) were also resistant to treatment in this model. Four strains susceptible in vitro were susceptible in this model. Two strains were misclassified by the MIC (Fig. 5). Strain (g) was susceptible in vitro but failed to respond in vivo. Strain (a), resistant to natamycin according to the MIC, was susceptible in both models of infection. This strain was also resistant in vitro to amphotericin B and failed to respond to treatment with amphotericin B in vivo.

**Discussion**

In a previous study, the authors demonstrated the correlation between the in vitro susceptibility of a series
of *C. albicans* clinical strains and the response in vivo to amphotericin B and natamycin. The panel of strains separated into two discrete populations on the basis of susceptibility in vivo correlating with in vitro susceptibility as determined by the tube dilution method. A similar, but less perfect, relationship was observed with natamycin. For both drugs, two other methods of testing in vitro susceptibility correlated less well with in vivo response than the tube dilution MIC.

The model of infection in the superficial cornea used in this and preceding experiments is attractive. The 48-hr period of treatment makes it convenient for these drug studies. However, there is some concern as to its similarity to human disease. The infection is confined to the superficial cornea and appears relatively self-limited so that prolonged treatment periods are not practical. Yeasts, largely in the blastospore phase, comprise the inoculum, and it is likely that transformation to a predominately hyphal phase with invasion of tissue has still not occurred 1 hr later when treatment begins. In human yeast infection, tissue invasion is characterized by the presence of pseudohyphae and true septate hyphae. Thus, it might be argued that instituting antifungal therapy to recently inoculated blastospores in the superficial cornea before infection is fully established does not reliably parallel human keratomycosis.

In the model of infection in the deep corneal stroma, the demonstration of blastospore transformation and tissue invasion at 24 hr supports the strategy of delaying treatment. The authors have shown previously that the infection is still active 9 days after inoculation, so that even with a delay of 24 hr, a period of at least 7 days is available for treatment. The model is also applicable to a broad series of strains as judged by the almost uniformly high isolate recovery rates in the control eyes in these studies. Thus, despite the logistic difficulties associated with the more prolonged treatment period, the authors believe that this model of experimental infection is useful and should be applicable to other therapeutic studies.

The present series of experiments confirm the findings with amphotericin B derived from the immediate treatment studies. The same two populations of strains are present with a sharp differentiation in vivo between the "resistant" and "susceptible" groups. A therapeutic effect was obtained for all susceptible organisms without resorting to a more intensive rate of drug administration despite the fact that the infection was more firmly established in the cornea because of the 24-hr delay in initiating treatment. The response amongst the susceptible strains varied, as in the early study. However, the range was broader—from 4.9–12.10 Δ log2 CFU—for the 0.15% amphotericin B treatment.

Classification of this panel of strains on the basis of the in vitro and in vivo response invites the description of the two populations as susceptible and resistant. However, it is still unclear whether this description is fully appropriate because the therapeutic regimen may not be optimal. It is conceivable that more intensive treatment could result in a therapeutic effect being observed with strains unresponsive to amphotericin B in this study. Among the susceptible group, the wide range of response is intriguing.

There are several possible explanations for the variability uncovered with these strains, including suboptimal therapy or differences in susceptibility among susceptible strains. Further studies are needed to ex-
amine this phenomenon and may point to the existence of a further subset of susceptible strains.

The response to natamycin was in sharp contrast to that observed with amphotericin B in several important respects. Strains susceptible to the drug administered 10 times a day in the model of immediate treatment became unresponsive to any but the most intensive regimen over the 5-day period. Thus, when institution of therapy was delayed 1 day, none of the strains were susceptible to natamycin given every hour—even when administration was on a 24-hr basis for the 5 days. It was only when the rate of administration was increased to every 30 min that a therapeutic effect was observed. In five of six strains the reduction in disease, as measured by recovery rates, was significant though modest when compared with the effect following amphotericin B administration. One strain noted to be susceptible in vitro and to immediate treatment was unresponsive in this model. This, and one other strain misclassified as resistant in vitro, reflects the less than perfect concordance between in vitro and in vivo susceptibility observed in the previous study with this drug.

As was the case with amphotericin B, the phase of the organism at the time treatment began appears to affect the response. However, in this case the difference is marked, appearing to indicate a reduction in susceptibility that applied to each strain previously responsive to treatment.

Among factors to be considered as responsible alone or in combination for this reduction in efficacy are differences between the models apart from the growth phase of the inoculum, a true difference in susceptibility between blastospores and hyphal phase organisms, and the development of resistance in previously susceptible strains. Although it is true that there is an important distinction as far as the inoculation site in the cornea is concerned, and therefore conceivably access to adequate levels of natamycin, results of other experiments involving immediate treatment with infections situated deep within the corneal stroma lead the authors to believe that other factors may be involved. In a previous study with the LV strain (identified as “h” in these experiments), an identical infection protocol was followed, but treatment was begun 1 hr after inoculation and administered 10 times a day for 5 days. A therapeutic response, equivalent to that seen in the model of superficial corneal infection, was seen. Yet, when the same strain was used but treatment was delayed, no therapeutic effect was present.

The entire series of experiments with these strains have been performed sequentially over a period of several years. It might, therefore, be argued that over this time resistance to the polynes might develop because of mutation during laboratory passage. The strains in this study were maintained at −70°C and were not passaged.

Another possible source of error in the authors’ method is the presence of residual drug in the excised corneas sufficient to inhibit organism growth during isolate recovery. The pharmacokinetics of topical amphotericin B and natamycin are not well elucidated so that the possibility of residual drug effects exists. Amphotericin B is known to bind to lipoprotein rapidly when administered systemically, leaving only about 5% of drug in a bioactive form. In the cornea after topical administration, approximately the same fraction of total drug present appears bioactive. Given the low levels of total drug measured in the cornea following topical administration, it appears unlikely that residual drug effects with amphotericin B are present. For natamycin the issue is less clear. Although recent studies have shown corneal concentrations of natamycin to be higher than amphotericin B following topical administration, the fraction that is in bioactive form is unknown. Nonetheless, it is unlikely that such an error was actually present for the following reasons: (1) the authors purposely delayed harvesting for 18 hr following the last treatment to allow for drug washout from the cornea; (2) this method of isolate recovery ensures a minimum dilution factor of 30 of any residual drug present; (3) the authors plate out the specimens at two further 1:10 dilutions, so that a dilution factor of 3,000 is possible; and (4) one of the strains (g) susceptible in vitro was fully resistant in vivo, isolate recovery rates being equivalent to those obtained from untreated controls.

This experiment was designed to evaluate the correlation between in vitro susceptibility and in vivo efficacy previously observed to amphotericin B and natamycin. It confirms, in a model more akin to human disease, the findings observed in the authors’ previous study. The concordance between the in vitro and in vivo response for this series of Candida albicans strains is good preliminary evidence for the value of the tube dilution method. A prospective evaluation in human infection may be the next appropriate step.

Key words: amphotericin B, natamycin, in vivo, in vitro, correlation, Candida albicans, keratitis, blastospores, hyphae

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References


