Influence of the Corneal Epithelium on the Efficacy of Topical Antifungal Agents

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A model of deep stromal Candida albicans infection was established by injecting 25 μl of a suspension containing 5 × 10⁹ colony forming units/ml of the yeast into corneas of pigmented rabbits. In this model, the infection lasts for more than 8 days. Using quantitative techniques, the authors compared the efficacy of six topical antifungal agents in the presence of an intact epithelium and in corneas debrided of epithelium. In corneas debrided on a daily basis, the polyenes (amphotericin B 0.15% and 0.075% and natamycin 5%) exhibited a significant antifungal effect. When the epithelium was left intact, 5% natamycin and 0.075% amphotericin B were without effect, while the efficacy of the 0.15% preparation of amphotericin B was much reduced. Removal of the epithelium appeared to affect adversely the efficacy of flucytosine. The imidazoles were not efficacious in this model. Invest Ophthalmol Vis Sci 25:855-859, 1984

Very little is known of the pharmacokinetics of topically applied antifungal agents. Most of these compounds are poorly soluble and, although clinical studies have supported their efficacy to varying degrees, corneal penetration and bioavailability are largely unknown.1-3 In previous animal experiments, we ranked these compounds in terms of their efficacy and evaluated the adverse effect of topical corticosteroid on their antifungal activity.4,5 A model of superficial infection was employed in each of these experiments. In this present study, we explored the efficacy of topical antifungal agents in the treatment of a deep corneal infection with particular emphasis on the barrier role of the epithelium.

Materials and Methods

Animal Model

Inoculum: A culture of Candida albicans (strain LV) grown for two days on trypticase soy agar with 5% sheep blood (BBL) was used to infect the corneas of outbred pigmented rabbits (weighing 1.5-3.0 kg). The minimal inhibitory concentrations of the various antifungal agents for this strain are as follows (μg/ml): amphotericin B 0.20; natamycin 6.25; flucytosine 0.39; miconazole 12.5; ketoconazole >50; econazole 25.4,6

Infection protocol: Rabbits were anesthetized with intramuscular Ketamine hydrochloride. A retrobulbar injection of 1% Xylocaine hydrochloride was given. Topical anesthesia was achieved with Opthaine 0.5%. The eye was propsected gently and, 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 in normal saline in a concentration of 5 × 10⁹/ml were injected and the needle was removed. If penetration of the anterior chamber occurred, the animal was removed from the study. Our animal utilization conforms to the ARVO Resolution on the Use of Animals in Research.

Efficacy of Topical Antifungal Agents

Agents evaluated: (1) Polyenes—Amphotericin B 0.15% and 0.075% (Fungizone, E.R. Squibb & Son; Princeton, NJ); Natamycin 5% suspension (Alcon Laboratories; Fort Worth, TX). (2) Imidazoles—Miconazole base 1% in Cremorph (Janssen Pharmaceuticals; New Brunswick, NJ); Miconazole nitrate 1% in polyethylene glycol 400 (Janssen); Ketoconazole 1% in polyethylene glycol 400 (Janssen); Econazole 1% in polyethylene glycol 400 (Janssen). (3) Pyrimidine—Flucytosine 1% solution in normal saline (Roche Laboratory; Nutley, NJ).

Study design: Following inoculation, the animals were separated randomly into three equal groups. All animals in the first two groups received the same antifungal therapy with both eyes in each animal undergoing treatment. The third group served as untreated controls. In Group 1, a 7-mm disc of epithelium was marked by a disposable trephine and gently removed by scraping with a #15 Bard Parker blade (Bard Parker Co.; Rutherford, NJ). The corneal epithelium
was left intact in the second treatment group, and also in the control group because preliminary experiments demonstrated that removal of the epithelium had no significant effect on quantitative isolate recovery in untreated animals. Beginning 1 hr after inoculation, the antifungal agent was administered hourly 10 times a day for 5 days. With selected drugs, the period was extended to 8 days. Each day, epithelial debridement was repeated and the corneas of the rabbits with intact epithelium were inspected carefully for evidence of epithelial loss. Eighteen hours after the conclusion of the treatment period, the animals were killed with commercially prepared euthanasia solution T-61 (Taylor Pharmacal; Decatur, IL).

Isolate recovery: The whole cornea was removed by excision at the limbus and cut into small pieces. These were then ground in a tissue grinder (Ultraturax Model SDT; Tekmar Co.; Cincinnati, OH) for three 10-sec intervals in 3 ml of sterile phosphate buffered saline. One-, ten-, and one hundred-microliter aliquots of each corneal suspension were plated in triplicate on trypticase soy agar with 5% sheep blood. After 48 hr incubation at 25°C, the colony forming units were counted and the number of CFU per cornea, based on a total volume of 3 ml, was calculated.

Statistical analysis: The measure of response to treatment was the logarithm to the base 2 of CFU. This transformation is required because the effect of treatment is proportional to pretreatment CFU. Since zero CFU counts were observed in some treatment groups, the actual response measure was log₂ (1 + CFU). The rationale for the log transformation is discussed extensively by Snedecor and Cochran, and Steel and Torrie.²⁸

In the basic experimental design, there were three groups (control, intact epithelium, absent epithelium) with four rabbits in each group. If the variability of an experiment exceeded certain statistical criteria (a standard error within an experimental group of greater than 1 in conjunction with a nonsignificant mean improvement of greater than 1 log₂ CFU in one of the treatment groups over the controls), we replicated the experiment to achieve greater precision. Data from all experiments were included in the subsequent analyses.

The data were analyzed using analysis of variance with subsampling.²⁸ This technique recognizes the fact that the rabbit is the basic experimental unit; all conclusions are based upon the numbers of rabbits, not upon numbers of eyes. However, it utilizes the additional information from the two eye measurements to produce a more precise measurement of the effect of the treatment upon a single rabbit, as well as an estimate of the intrarabbit, intereye variability. This technique is described in detail in statistical texts.²⁸

Each hypothesis of interest was tested using a single degree of freedom contrast,²⁸ which compared the particular experimental groups. For replicated experiments, the contrast was constructed so that each experimental group was compared with the control from the same experiment. For example, intact epithelium group (EPION) versus control (CON) would be tested with a contrast of the form:

\[(EPION_1 - CON_1) + (EPION_2 - CON_2).\]

In this notation the subscript “1” denotes the first performance of the experiment; “2” the second performance.

The alternative of simply pooling the data is unacceptable because the level of disease in the control group may be different for different replications of the same experiment.

For single experiments, the estimated standard deviation of a contrast was derived from the between-rabbits mean square from the analysis of variance with subsampling. For replicated experiments, it was derived from the between-(rabbits by replications) mean square. This accounted for the fact that rabbits were the basic experimental units, not eyes.

Results

Animal Model

The inoculation of 25 μl of a 5 × 10⁹ ml suspension of C. albicans (strain LV) produced a moderately severe keratitis that lasted for at least one week. Clinically, the corneas became progressively more inflamed over this period. Daily examination with fluorescein revealed that the corneal epithelium remained undisturbed in both untreated control animals and those treated without epithelial debridement. Histologic studies demonstrated the presence of extensive mycelial phase growth within the stroma (Fig. 1) accompanied by inflammatory cell infiltration confirming the active nature of the infection. The uniform isolate recovery rate from the untreated controls at both 5 and 8 days is indicative of the consistency of the model (Table 1). It also parallels the clinical observation of an active keratitis. Even after 8 days, an average of 65,000 CFU were isolated from the untreated control corneas.

Efficacy of Drug Therapy

Amphotericin B: With both concentrations of amphotericin B (0.15% and 0.075%), a highly significant therapeutic effect was noted in corneas debrided of epithelium (Table 1, Fig. 2). When the epithelium was left intact a significant, though much reduced, antifungal effect was present with the 0.15% concentration. No significant effect was observed in animals with intact corneal epithelium for the 0.075% preparation.

Natamycin: Natamycin 5% was significantly efficacious only in corneas debrided of epithelium. In this case, a mean reduction of 4.76 log₂ CFU was observed (Fig. 3, Table 1).
Flucytosine: After 5 days of treatment with flucytosine, the corneas with intact epithelium contained fewer organisms than debrided corneas and untreated controls. The difference, however, was not statistically significant. When the period of treatment was extended to 8 days, the reduction, although small, became significant (Fig. 4).

Imidazoles: A therapeutic effect was not apparent with any of the imidazoles, even when, as in the case of miconazole, the treatment period was extended to 8 days (Table 1).

Discussion

In previous experiments with a quantitative model of *C. albicans* infection in the rabbit cornea, we found that treatment with amphotericin B was superior to natamycin, flucytosine, and imidazoles, even when amphotericin B was used in concentrations as low as 0.075%. Because the model is one of superficial infection, the influence of factors such as corneal penetration and bioavailability is minimal. Thus, it is possible that these observations may not be applicable to deep fungal invasion of the cornea.

In the present model, the inoculum is deposited deep within the corneal stroma and produces a persistent infection. Moreover, the epithelium remains undisturbed. In this model, it is possible to study antifungal agents under conditions that more closely parallel human disease.

Our data support the impression gained from clinical studies that both amphotericin B and natamycin are highly active in deep corneal infections when applied topically.9-11 The excellent response in corneas debrided of epithelium suggests that both of these polyenes easily penetrate the corneal stroma. It is also clear that an intact epithelial layer is a barrier to stromal penetration of antifungal agents.

Table 1. Quantitative isolate recovery rate following antifungal treatment of *C. albicans* keratitis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Untreated controls</th>
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<tbody>
<tr>
<td></td>
<td>N*</td>
<td>Mean†</td>
<td>SE‡</td>
<td>Range</td>
<td>N*</td>
<td>Mean</td>
<td>SE</td>
<td>Range</td>
<td>N*</td>
<td>Mean</td>
<td>SE</td>
<td>Range</td>
<td>N*</td>
<td>Mean</td>
<td>SE</td>
<td>Range</td>
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<tr>
<td>Amphotericin B</td>
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<td></td>
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<tr>
<td>0.15% (5 days)</td>
<td>8</td>
<td>15.85</td>
<td>0.354</td>
<td>13.4-17.0</td>
<td>8</td>
<td>12.83</td>
<td>0.863</td>
<td>9.6-15.3</td>
<td>8</td>
<td>4.50</td>
<td>1.30</td>
<td>0-10.8</td>
<td>0.03</td>
<td>&lt;0.0001</td>
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<td>Amphotericin B</td>
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<tr>
<td>0.075% (5 days)</td>
<td>8</td>
<td>16.59</td>
<td>0.235</td>
<td>14.9-17.6</td>
<td>8</td>
<td>15.52</td>
<td>0.328</td>
<td>12.8-16.7</td>
<td>8</td>
<td>5.70</td>
<td>1.24</td>
<td>1.8-14.9</td>
<td>0.20</td>
<td>&lt;0.0001</td>
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<td>Natamycin 3% (5 days)</td>
<td>4</td>
<td>16.27</td>
<td>0.110</td>
<td>16.0-16.5</td>
<td>4</td>
<td>1.51</td>
<td>0.143</td>
<td>15.2-15.8</td>
<td>4</td>
<td>11.51</td>
<td>0.478</td>
<td>10.5-12.7</td>
<td>0.10</td>
<td>&lt;0.0001</td>
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<td>Fluconazole 1% (5 days)</td>
<td>4</td>
<td>16.52</td>
<td>0.252</td>
<td>15.9-17.1</td>
<td>3</td>
<td>15.37</td>
<td>0.188</td>
<td>15.0-15.6</td>
<td>4</td>
<td>16.61</td>
<td>0.609</td>
<td>14.8-17.4</td>
<td>0.11</td>
<td>&gt;0.20</td>
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<td>Fluconazole 1% (8 days)</td>
<td>8</td>
<td>15.65</td>
<td>0.631</td>
<td>13.4-18.1</td>
<td>8</td>
<td>12.81</td>
<td>0.955</td>
<td>5.8-17.4</td>
<td>8</td>
<td>14.53</td>
<td>0.710</td>
<td>12.2-17.9</td>
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<td>Miconazole 1% (5 days)</td>
<td>4</td>
<td>15.91</td>
<td>0.736</td>
<td>14.4-17.3</td>
<td>4</td>
<td>15.62</td>
<td>0.471</td>
<td>14.4-16.6</td>
<td>4</td>
<td>15.02</td>
<td>0.577</td>
<td>13.5-16.2</td>
<td>&gt;0.20</td>
<td>&gt;0.20</td>
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<td>Miconazole 1% (8 days)</td>
<td>4</td>
<td>15.97</td>
<td>1.51</td>
<td>11.8-18.4</td>
<td>4</td>
<td>18.04</td>
<td>0.859</td>
<td>15.5-19.1</td>
<td>4</td>
<td>15.12</td>
<td>1.60</td>
<td>11.5-18.2</td>
<td>&gt;0.20</td>
<td>&gt;0.20</td>
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<td>Miconazole nitrate 1% (5 days)</td>
<td>4</td>
<td>16.72</td>
<td>0.480</td>
<td>15.6-17.6</td>
<td>3</td>
<td>16.12</td>
<td>0.486</td>
<td>15.2-16.8</td>
<td>4</td>
<td>17.51</td>
<td>0.321</td>
<td>16.6-18.2</td>
<td>&gt;0.20</td>
<td>&gt;0.20</td>
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<tr>
<td>Econazole 1% (5 days)</td>
<td>3</td>
<td>15.22</td>
<td>0.247</td>
<td>14.7-15.6</td>
<td>3</td>
<td>15.43</td>
<td>0.363</td>
<td>14.8-16.1</td>
<td>3</td>
<td>16.15</td>
<td>0.528</td>
<td>15.5-17.2</td>
<td>&gt;0.20</td>
<td>0.15</td>
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<tr>
<td>Ketoconazole 1% (5 days)</td>
<td>3</td>
<td>15.47</td>
<td>1.12</td>
<td>8.1-17.8</td>
<td>7</td>
<td>13.95</td>
<td>0.765</td>
<td>10.7-16.5</td>
<td>8</td>
<td>15.75</td>
<td>0.321</td>
<td>13.5-17.4</td>
<td>&gt;0.20</td>
<td>&gt;0.20</td>
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* number of animals in each group.
† log colony forming units.
‡ standard error.

Fig. 1. *C. albicans* keratitis in the rabbit on day 3 following inoculation. The inoculum has been deposited in the midstroma. Active mycelial phase growth is occurring. The corneal epithelium is intact over the lesion. (PAS stain, X300).

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penetration of both drugs. We were unable to demonstrate efficacy with either 5% natamycin or 0.075% amphotericin B if the corneal epithelium was left intact. Amphotericin B and natamycin are both insoluble in lipid, so the phenomenon is not unexpected. The barrier effect, however, is not absolute, since the 0.15% concentration of amphotericin B exhibited a significant although much reduced therapeutic effect even in the presence of the epithelial layer. While this result merely may reflect a concentration effect with this drug, it should be noted that amphotericin B has been shown to enhance the water permeability of the corneal epithelium. Such an effect might permit subsequent applications of the aqueous colloidal suspension of the drug to penetrate the cornea. There are no reports in the literature of amphotericin B levels in the cornea after topical administration but Jones, using a double-labeling technique, reported peak levels of 8 μg/ml in aqueous after combined topical administration of 0.5% amphotericin and subconjunctival injection of 2 mg. Our own studies of amphotericin B levels in the cornea confirm the barrier role of the corneal epithelium.

The efficacy of amphotericin B found in the present study even in the 0.075% strength suggests that therapeutic concentrations within the cornea may be achievable easily by the topical route using preparations that are more dilute than those currently advocated. This is important because one of the major obstacles to topical use of amphotericin B has been the severe corneal toxicity associated with more concentrated preparations.

Natamycin is insoluble in water and is prepared for clinical use as a 5% microfine aqueous suspension. Although there is good clinical evidence for its efficacy, there are no published studies of corneal penetration or optimal topical formulation. It is not yet clear how active drug enters the corneal stroma from this particulate suspension, but diffusion facilitated by prolonged contact time may be one possibility.

Flucytosine exhibited somewhat different charac-

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**Fig. 2.** The influence of the corneal epithelium on the efficacy of topical amphotericin B (0.15% and 0.075%) in the treatment of *Candida keratitis* as measured by the number of colony forming units/cornea recovered after 5 days of treatment (mean ± standard error). (P1 = untreated controls with intact epithelium versus treated animals with intact epithelium. P2 = untreated controls with intact epithelium versus treated animals with debrided epithelium. N = number of animals in each group).

**Fig. 3.** Influence of the corneal epithelium on the efficacy of topical 5% natamycin as measured by the number of colony forming units/cornea recovered after 5 days of treatment (mean ± standard error). (P1 = untreated controls with intact epithelium versus treated animals with intact epithelium. P2 = untreated controls with intact epithelium versus treated animals with debrided epithelium. N = number of animals in each group).

**Fig. 4.** The influence of the corneal epithelium on the efficacy of topical 1% flucytosine in the treatment of *C. albicans* keratitis in rabbits as measured by the number of colony forming units/cornea recovered after 5 and 8 days of treatment (mean ± standard error). (P1 = untreated controls with intact epithelium versus treated animals with intact epithelium. P2 = untreated controls with intact epithelium versus treated animals with debrided epithelium. N = number of animals in each group).
teristics in this model. Efficacy was not enhanced by removal of the epithelium. Instead, greater reduction of disease occurred in the corneas with intact epithelium. However, this effect was not marked and, although significant, was achieved only after a prolonged period of treatment. It did not approach that of amphotericin B even though the strain is extremely susceptible to both agents in vitro. While this disparity may be indicative of pharmacokinetic differences between the polyenes and flucytosine relating to drug penetration and bioavailability within the corneal stroma, we also noted a similar relative efficacy in the model of superficial infection where such factors are probably less important.4,5 Since amphotericin B is fungicidal in vitro against this strain4,5 and flucytosine is fungistatic, it is tempting to conclude that one factor responsible for the disparity in the in vivo efficacy of the two drugs may be this difference in their antifungal mechanisms.

The poor performance of the imidazoles as a group did not allow an evaluation of the effect of the corneal epithelium on their efficacy. The failure of ketoconazole was not unexpected since we had been unable to demonstrate a significant antifungal effect with it even for superficial infections.4 Econazole also was ineffective, but, again, this result was not surprising for the same reason (O'Day, unpublished data). The disappointing results with miconazole, however, are more difficult to interpret, since reports in the literature support the value of topical miconazole in clinical cases.16,17

The minimal inhibitory concentration of miconazole for the LV strain used in these studies was 12.5 μg/ml.4 Thus, the failure of miconazole might be attributable to strain resistance. We previously have shown miconazole to be effective against this strain in the model of superficial infection, although its efficacy was far inferior to that of the polyanes and easily negated by concurrent corticosteroid administration.4,5 It is reasonable to presume that drug penetration in superficial infections is a far less critical factor in determining efficacy than in a deep stromal infection. For this reason, a superficial infection offers the better opportunity for an agent to display efficacy. However, Foster and Stefanyszyn have reported high drug levels in the cornea and anterior chamber after topical administration.5 How much of this drug is in a bioavailable form is not known since the method used measured the total amount of miconazole. Experiments with additional strains and an estimation of bioavailable drug levels in the cornea may help explain the apparent lack of efficacy of miconazole.

The data from our experiments support the validity of this model of deep stromal C. albicans infection in outbred rabbits. They show that the disease produced in normal rabbit eyes is consistent and the result of tissue invasion by the inoculated yeast. We believe, based on our present studies, that manipulation of this model under the appropriate conditions can yield important new information in an area of therapy that is, as yet, poorly understood. Indeed, the observations concerning the impact of the epithelium on the efficacy of the polyenes and flucytosine have considerable clinical implications that merit further investigation.

Key words: Candida albicans, keratitis, corneal epithelium, antifungal agents, efficacy

References