ORALLY ADMINISTERED ANTIFUNGAL THERAPY FOR EXPERIMENTAL KERATOMYCOSIS*

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INTRODUCTION

CURRENT STRATEGIES FOR THE TREATMENT OF FUNGAL CORNEAL INFECTIONS rely on locally administered antifungal agents, usually by the topical route.[1-4] Although certain antifungal agents can be given by subconjunctival injection, toxicity is a severe problem particularly with the polyenes.[1,2] The toxicity of most systemic antifungal agents as well as presumed poor corneal penetration has also discouraged systemic use for nonlife-threatening infections such as keratomycosis.[1,2]

Keratomycosis is prevalent in parts of the world where specialized medical care tends to be least available.[2,5,6] Although topically administered agents, most notably natamycin,[1,3,4,7] are known to be efficacious in the treatment of fungal keratitis, the high cost of these drugs and the requirement for a prolonged period of administration are practical obstacles to widespread use. Even in the United States, such factors may inhibit successful treatment. Moreover, the relatively small number of cases of keratomycosis worldwide, compared to systemic mycoses, provides little incentive to drug companies to develop topical agents that would be available at reasonable cost.

The notion of treating keratomycosis with orally administered agents appear to offer a partial solution to this problem provided the disadvantages of toxicity and poor ocular penetration can be overcome. By using agents developed for the treatment of systemic mycoses, cost may be significantly lowered so that availability can become a practical possibility, even in economically disadvantaged parts of the world.

This approach has received little attention in the literature. Two studies from India[8,9] report encouraging results with oral antifungal therapy in

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patients with infections caused by diverse fungi. Although these and a few isolated case reports[10-12] appear to encompass the sum of clinical experience to date, they clearly support the merit of exploring this concept further.

Recently the triazoles, a new group of antifungal agents, have become available.[13,14] These compounds are active when given by mouth and are considerably less toxic than current antifungal agents. They appear suitable candidates for use in the systemic therapy of experimental keratomycosis.

Three genera (*Fusarium, Aspergillus* and *Candida*) have emerged as important pathogens in the cause of fungal keratitis worldwide.[2,5,6] While there has been some success in infecting the corneas of experimental animals with *Candida*[15-17] and *Aspergillus*,[18,19] fusarial infection has been virtually impossible to establish without extensive host modification.[20] The following studies of systemic therapy, therefore, concern the treatment of experimental *Candida* and *Aspergillus* keratitis.

BACKGROUND

Inasmuch as the corneas of most experimental animals appear naturally resistant to fungal infection, the development of animal models of fungal keratitis has proven to be a difficult task.[20-22] Even when infection is established, it tends to be short-lived so that the interval available for experimental therapeutic studies is relatively brief.[16,17,19,20] Nevertheless, animal models play an important role in the elucidation of therapy, though novel strategies may be required to demonstrate a therapeutic effect.[23,24]

Candida albicans has been the organism most commonly utilized. Two different models have been devised — an infection in the superficial corneal stroma[23,25] suitable for short-term studies and a model of more prolonged deep stromal infection.[15-17,26]

To produce an infection with *Candida* in the superficial cornea, fungal elements, chiefly blastoconidia, are inoculated into the previously abraded or trephined corneal surface.[23,25] Deep infection is established by direct injection of a standardized inoculum into the midcorneal stroma.[15-17,26,27]

Measuring progress of the disease and therapeutic response is hampered by the indolent nature of the infection. Nonparametric methods for scoring clinical signs have been devised.[15,23,26,28] A disadvantage with this approach, however, is its essentially empirical nature. Since the signs reflect the intensity of the host response to several different inflammatory

stimuli, the precise relationship to the actual number of viable organisms is uncertain. In an effort to refine these observations, some investigators have attempted to use isolate recovery from the surface of the cornea[15,23] or from the whole cornea at the conclusion of the experiment[26] as a measure, but again reliability of this approach is questionable.

Quantitative isolate recovery techniques, in general use for the study of infectious processes in other organ systems, have been more recently introduced to the study of ocular infections caused by yeasts.[16,17,25,29] They appear to offer a precise and sensitive method for measuring therapeutic response provided the experiment is appropriately designed and statistically analyzed.[24] Using this methodology, comparative studies of infections involving multiple strains also become possible.[30,31]

In normal rabbits, the duration of disease, when defined as the period during which therapy can be usefully studied, has been reported to range from about 5 to 10 days following inoculation.[17] Beyond this period the disease begins to resolve spontaneously and variance in isolate recovery rates from individual corneas increases, thus rendering detection of a therapeutic effect difficult. However, if the period of experimentation is confined to the active disease interval, valid therapeutic studies can be performed.

Several investigators have used corticosteroid administration to render the animal more susceptible to candidal infection and thus prolong the disease.[15,26,28] However, this practice is probably unnecessary.[25,27] In fact, experimental *Candida* infection in the rabbit cornea has been used to study the effect of steroid administration on fungal keratitis as well as on the antifungal activity of various antimycotic agents.[32]

Establishing infections with molds in animal corneas is more difficult. Despite efforts over the years by many investigators who recognized the importance of such models, success has been elusive. In addition to the problem of establishing an infection of sufficient duration for study, a satisfactory method for evaluating therapeutic response remains to be defined.

Previous investigations have relied mostly on the scoring of clinical signs without attempting to correlate these signs with mycologic evidence of infection.[19] An objective method of estimating the amount of viable fungal elements would be desirable. However, in the presence of hyphae, a potential source of error is introduced when quantitative techniques are applied to infections with molds. Tissue fragmentation is an essential component of this technique. If its effect on hyphae is not uniform, the number of colony forming units (CFU) recovered which form the basis of analysis may be highly variable and erroneous. Experiments of the type

described for *Candida*,[16,17,25,29] which have helped to validate the technique for yeast infections, have yet to be reported for infections involving molds. The technique has been used by only one other investigator.[18]

Early investigators, attempting to develop corneal infections with a variety of fungi, frequently resorted to steroid administration to enhance the disease.[20-22] For *Fusarium solani* in particular this has proved to be the only way to establish a clinical infection.[20] Infecting animal corneas with *Aspergillus sp.* has also proved difficult. Ellison and associates[28] developed a model of *Aspergillus fumigatus* keratitis in rabbits that appeared to last 2 weeks. Unfortunately, the investigators, in these and subsequent experiments,[33] relied on clinical evaluation without attempting quantitative mycological correlations. deLomas and associates[19] also described the production of an infection that was clinically apparent for 2 weeks, but again there was no quantitative mycological confirmation. A model developed by Komadina and associates[18] was evaluated quantitatively and found to persist for at least 5 days. However, the counts were quite variable and the investigators found it difficult to establish consistent disease. Steroid administration was a prerequisite for establishing disease in these animals.

This difficulty in establishing fungal infections in animal corneas is usually attributed to inate resistance of the host as the major factor. However, it is also probable that strain-related factors play a part. In extensive studies, a wide variation in the ability of individual *Candida albicans* strains to colonize the rabbit cornea following injection of a standardized inoculum was noted[25] (author, unreported data, 1980-89). Strains of *Aspergillus fumigatus* also appear to vary in pathogenicity for the cornea. Pilot studies with several strains derived from clinical cases revealed a marked variation in duration and in intensity of disease (author, unpublished results, 1985-89). These observations may help explain the apparently conflicting experience of several investigators with regard to their success in establishing an infection. They provide support for continued efforts to identify strains that will produce a satisfactory infection without the use of steroid treatment.

Systemic therapy for experimental keratomycosis has undergone only very limited study. Oral ketoconazole was evaluated in a model of *Aspergillus fumigatus* keratitis in rabbits.[18] A therapeutic response after 5 days of treatment with ketoconazole alone could not be detected. However, in one experiment ketoconazole appeared to augment the effect of topical natamycin, though the effect was slight and could not be confirmed subsequently.[18] Ishibashi and Matsumoto,[15,34] in two separate studies of experimental *Candida albicans* keratitis, reported a significant

clinical response to oral ketoconazole and intravenous miconazole. Empirical clinical findings were used to score the disease in these animals with no attempt at mycologic validation. Even by these criteria the disease in untreated controls appeared very mild so the real significance of their observations is uncertain. Quantitative isolate recovery was not attempted at the conclusion of either study. The results of daily surface cultures were inconclusive.

Both ketoconazole and miconazole belong to the imidazole class of antifungal agents. They are considerably less toxic than polyene antifungals but are highly plasma protein bound.[35] Foster and Stefanyszyn[36] demonstrated good miconazole levels in the cornea when the drug was given to rabbits intravenously. Ketoconazole has also been detected in the cornea following systemic administration.[35,37]

The triazoles, a new group of agents derived from the imidazoles, show promising antifungal activity. Two members of the group, fluconazole and itraconazole, have received the most attention.[38,39] Fluconazole is water soluble and appears to have excellent systemic absorption when given orally.[14] The incidence of systemic side effects is very low.[35] It thus exhibits characteristics that are favorable for an oral antifungal agent for the treatment of keratomycoses. In contrast to the polyenes and the imidazoles, fluconazole has a low affinity for plasma proteins,[35] and preliminary studies indicate that the agent is well-distributed in body tissues including the brain.[40] When administered intravenously, high intraocular and corneal levels have been measured.[35] Fluconazole appears to have good anti-candidal activity.[35,38,41,42] Experimental studies also suggest activity against *Aspergillus*.[43]

Itraconazole, in contrast to fluconazole, is poorly soluble in water. Protein binding is high and tissue levels are inferior to those achieved with fluconazole.[35] The agent does not penetrate the cerebrospinal fluid in high concentration.[35] Despite the seemingly poor pharmacologic profile, itraconazole shows promise as a systemic antifungal agent in a number of different experimental fungal infections.[35,39,44] Thus, both fluconazole and itraconazole have potential as oral agents for keratomycosis. Fluconazole, because of its superior pharmacokinetics and good tissue levels following oral administration, was chosen as the oral antifungal agent for this study.

EXPERIMENTAL STUDIES

The studies that follow were designed to evaluate the efficacy of orally administered fluconazole in the treatment of experimental keratomycosis.

The first series of experiments (I and II) are devoted to the development of animal models of infection due to *Candida albicans* and *Aspergillus fumigatus*. Next (experiment III), the corneal uptake of orally administered fluconazole is investigated. Finally, in experiments IV and V, the response to treatment in these models is evaluated.

I. DEVELOPMENT OF A RABBIT MODEL OF *Aspergillus fumigatus* KERATITIS

EXPERIMENT I-A. INFLUENCE OF TISSUE HOMOGENIZING TIME ON ISOLATE RECOVERY FROM THE CORNEA

The purpose of this experiment was to examine the effect of tissue homogenization on isolate recovery from corneas infected with *Aspergillus fumigatus*.

MATERIALS AND METHODS

Inoculum

A strain of *Aspergillus fumigatus* (VE147) isolated from a patient with fungal keratitis was selected for these studies. In preliminary studies the fungus produced a progressive keratitis when inoculated into the corneas of Dutch-belted rabbits. It was maintained in culture on Sabouraud's dextrose agar at room temperature and periodically passaged. Prior to each experiment, the strain was inoculated onto a fresh plate of Sabouraud's dextrose agar and grown to confluence over 3 to 4 days. An inoculum, consisting predominately of spores but also containing small hyphal fragmetns, was harvested by loop and suspended in 15 ml normal saline containing 50 μl of Tween 80. After further dilution with normal saline to the desired percent transmission (T) using a spectrophotometer (Bausch & Lomb, Spectronic 20) set at 550 nm, the final concentration was verified by plate counting.

Animals

Dutch-belted rabbits of either sex (1.5 to 2.5 kg in weight) were used in these and subsequent experiments. They were handled in compliance with the standards set forth in the statement issued by the Association for Research in Vision and Ophthalmology for Research Using Animals.

Method of Inoculation

Five 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 100 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 inoculum, standardized to 90% T at 550 nm (29,700 CFU/ml) were injected and the needle was removed. If penetration of the anterior chamber occurred, the animal was removed from the study. Five days later the animals were sacrificed with commercially prepared euthanasia solution (T-61, Taylor Pharmacal, Decatur, IL).

In a second series of five rabbits, a similar protocol was followed with the same strain but with the inoculum standardized to 95% T at 550 nm. After 7 days, animals were sacrificed.

Tissue Fragmentation Technique

The corneas were excised at the limbus. To facilitate fragmentation, each was first cut into 16 to 20 small pieces of approximately equal size. These were then placed in a test tube containing 3 ml of buffered normal saline. Preliminary studies indicated that operating the tissue grinder (Ultra-Turrax Model SDT, Tekmar Co, Cincinnati, OH) for more than 10 seconds at a time caused perceptible warming of the tissue. Grinding periods were therefore limited to 10 seconds. In order to study the effect of tissue fragmentation on isolate recovery, the entire tissue from each cornea was ground for a total of 90 seconds in 10-second increments. After 30, 60, and 90 seconds, 0.1 ml of the whole ground cornea was removed and diluted in 0.9 ml phosphate buffered saline (PBS). Ten- and one hundred-microliter aliquots of the diluted sample were plated in triplicate onto Sabouraud's dextrose agar plates. One hundred microliters of the undiluted ground sample were also plated in triplicate on Sabouraud's dextrose agar. These aliquots represent 1, 10, and 100 μl, respectively, of the original ground sample. After incubation at 25°C for 48 hours, the CFU were counted for the dilution that provided a minimum of 10 colonies per plate. In each instance, this was the 100 μl aliquot. The number of CFUs/cornea was calculated for each grinding period.

RESULTS

The length of time used for tissue fragmentation in the isolate recovery technique did not appear to influence the isolate recovery rates in either experiment (Table I, inoculum 90% T and Table II, inoculum 95% T). For each cornea colony, the number of CFUs recovered from the samples were similar despite different grinding times. Variance in recovery rates amongst different corneas was also low regardless of the duration of the grinding period.

TABLE I: EFFECT OF TISSUE GRINDING TIME ON ISOLATE RECOVERY (log_2 CFU): FROM CORNEAS INFECTED WITH *Aspergillus fumigatus* (INOCULUM STANDARDIZED TO 90% T)

	NO. OF CFUs/CORNEA (log_2)				
	1	2	3	4	5
Sample at 30 seconds	9.82	10.02	9.80	10.51	10.28
Sample at 60 seconds	9.40	9.90	9.84	10.43	10.21
Sample at 90 seconds	9.64	10.07	9.86	10.54	10.19

TABLE II: EFFECT OF TISSUE GRINDING TIME ON ISOLATE RECOVERY (log_2 CFU): FROM CORNEAS INFECTED WITH *Aspergillus fumigatus* (INOCULUM STANDARDIZED TO 95% T)

	NO. OF CFUs/CORNEA (log_2)				
	6	7	8	9	10
Sample at 30 seconds	10.53	10.92	10.72	10.74	10.43
Sample at 60 seconds	10.46	10.80	10.60	10.72	10.43
Sample at 90 seconds	10.36	10.74	10.46	10.74	10.54

EXPERIMENT I-B. CLINICAL AND MICROBIOLOGIC EVALUATION OF EXPERIMENTAL *ASPERGILLUS FUMIGATUS* KERATITIS

In preliminary studies two strains (VE147 and VE148), standardized to 95% T at 550 nm, were injected intrastromally into corneas of Dutch-belted rabbits. Both were found to produce consistent quantitative isolate recovery rates. With strain VE147, the disease was short-lived and clinically mild. However, strain VE148 was associated with higher isolate recovery rates and a more prolonged clinical disease.

MATERIALS AND METHODS

Method of Inoculation

An inoculum of *Aspergillus fumigatus* (strain VE148), standardized to 95% T at 550 nm, was used to infect the right corneas of 45 Dutch-belted rabbits (1.5 to 2.5 kg). Prior to inoculation animals were assigned to one of three groups. The first was observed for 5 days, the second for 10 days and

the third for 15 days. There were 15 rabbits in each group. Each group of animals was sacrificed with T-61 euthanasia solution at the conclusion of the observation period.

Mycological Evaluation

Corneas were assayed for residual organisms using quantitative isolate recovery techniques as described in the previous experiment (I-A). A tissue fragmentation time of 30 seconds was employed for this and all subsequent experiments.

Clinical Evaluation

On days 2, 5, 10, and 15 following inoculation, the infected *Candida* were evaluated biomicroscopically by an observer masked as to the day of sacrifice (except on day 15). The following progressive grading system developed in preliminary studies was used (Figs 1 to 5):

0 = No disease is present.
1 = Minimal infiltrate and edema involve the inoculation site.
2 = Infiltrate surrounds inoculation site; a fine reticular pattern of filaments project into the adjacent stroma for less than half of the perimeter of the lesion.
3 = The reticular pattern of filamentous projections extend from more than half the perimeter of the lesion.
4 = The width of the lesion exceeds 50% of the horizontal corneal diameter. Iris vessels are injected.
5 = Hypopyon is present.

FIGURE 2
Experimental *Aspergillus fumigatus* keratitis: grade 2. Filamentous projections extend from less than half the perimeter of the lesion.

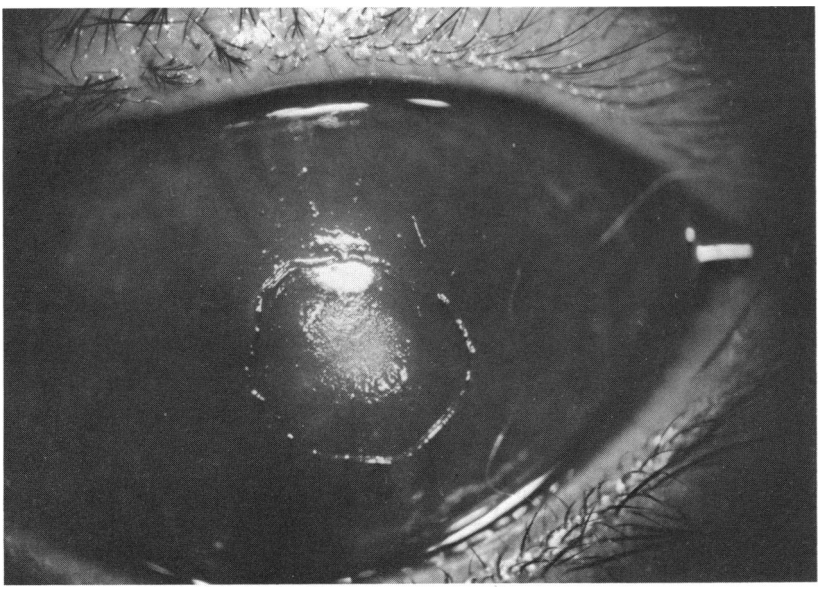
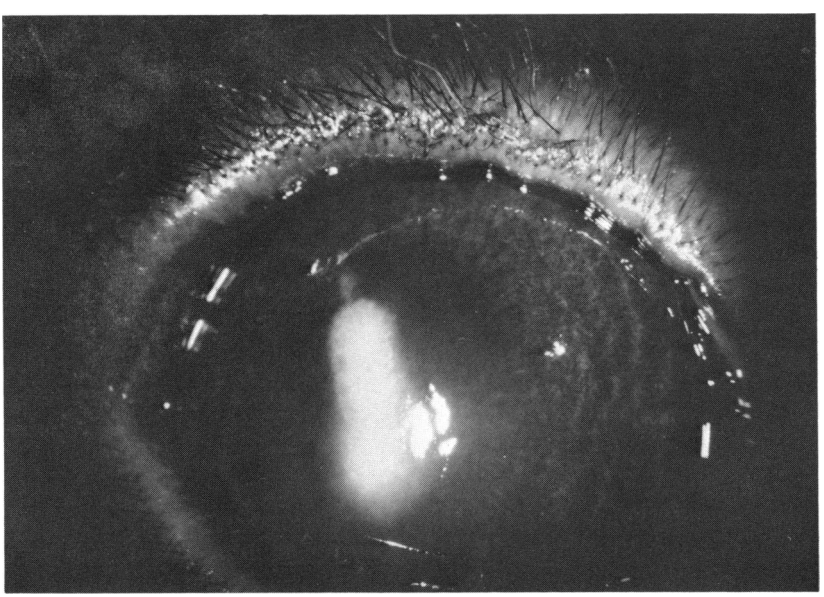


FIGURE 1
Experimental *Aspergillus fumigatus* keratitis: grade 1. There is minimal infiltrate and edema at inoculation site.



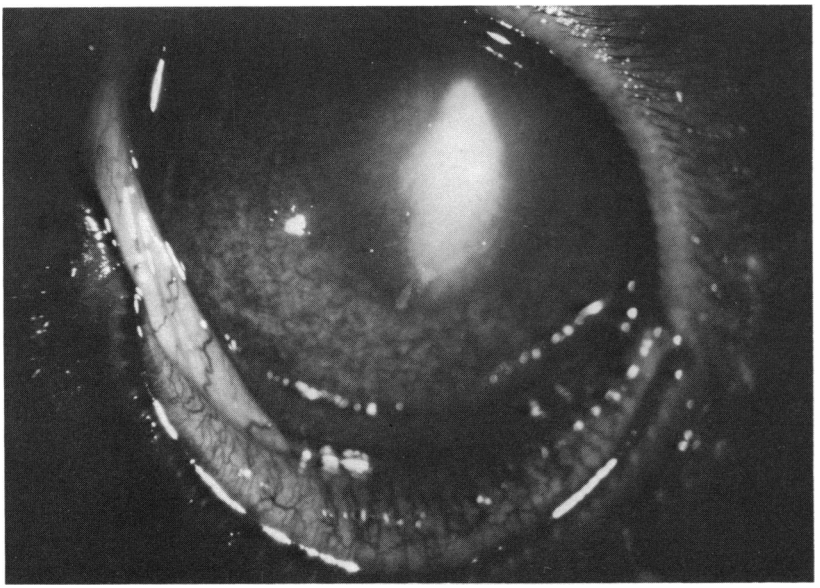
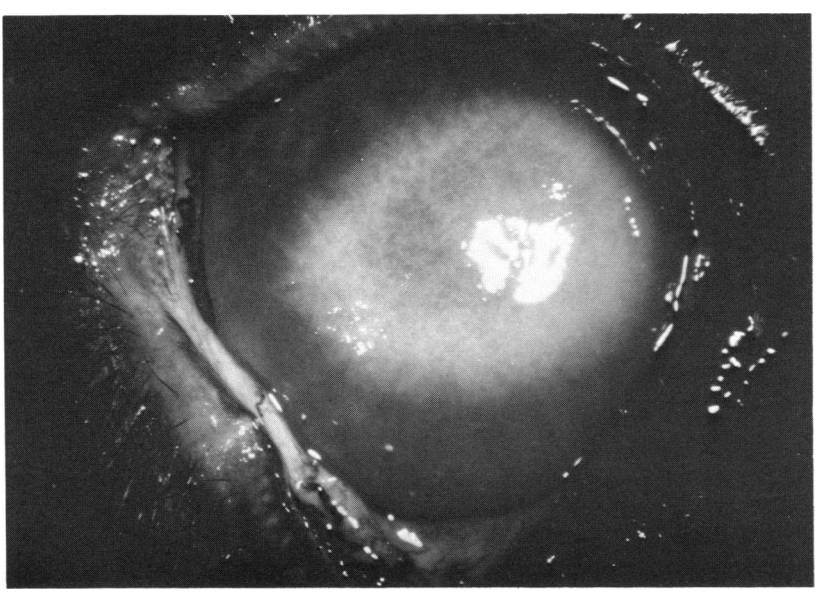


FIGURE 3
Experimental *Aspergillus fumigatus* keratitis: grade 3. Filamentous projections extend from more than half the perimeter of the lesion.



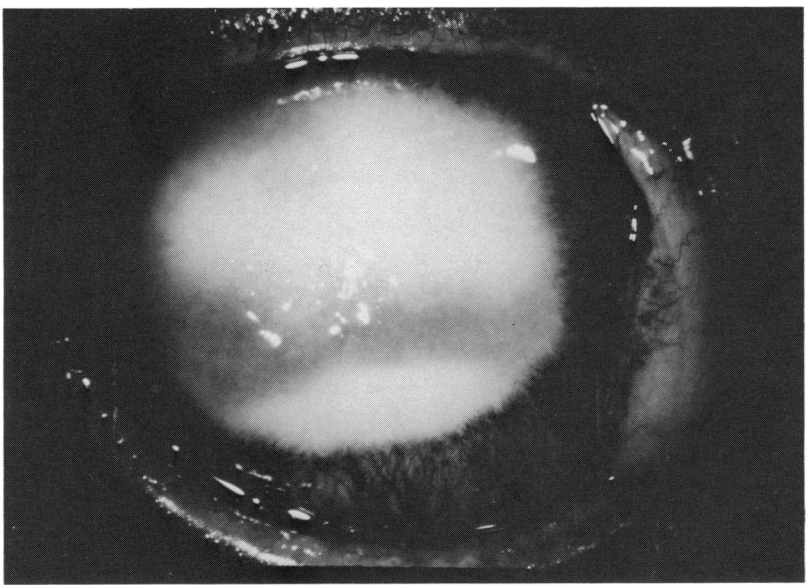


FIGURE 5
Experimental *Aspergillus fumigatus* keratitis: grade 5. The width of the lesion is greater than half of the corneal diameter; hypopyon is present.

FIGURE 4
Experimental *Aspergillus fumigatus* keratitis: grade 4. The width of the lesion is greater than half the corneal diameter.

RESULTS

Clinical Course

Inoculation of the corneas of Dutch-belted rabbits with *Aspergillus fumigatus* (strain VE148) produced an infection that persisted for at least 10 days. Two distinct clinical patterns were observed:

1. The earliest sign was the appearance of an infiltrate at the site of injection. By day 2, fine filamentous projections could be seen extending into the surrounding stroma from the central infiltrate. Over the next 13 days the intensity of the inflammation increased slowly in most animals as the size of the infiltrate enlarged and the filamentous projections became more apparent. Dilation of iris vessels was observed in some rabbits and an occasional hypopyon developed. Rarely, corneal ulceration occurred. No eyes perforated.
2. Minimal infiltrate was apparent at the site of injection on day 2. In some animals short filamentous projections were observed extending into the immediately adjacent clear stroma but they involved less than half of the perimeter of the lesion. Thereafter, the course was more variable. In most animals the inflammation tended to subside gradually after a transient increase in intensity around day 5. Occasionally a progressive inflammation with extensive invasion of the cornea and the development of hypopyon was noted by day 10.

Correlation Between Clinical Severity and Quantitative Isolate Recovery Rates in Corneas Infected with Aspergillus fumigatus

The average number of organisms isolated from these corneas showed a steady decrease over the period of observation from a high of 10.29 $\log_2$ CFU on day 5 to 2.89 $\log_2$ CFU on day 15 (Fig 6). The average clinical score, however, did not show a parallel reduction in severity, increasing slightly by day 10 before decreasing to 80% on the 5th day score on the 15th day. The course of the keratitis as measured by the clinical severity score correlated strongly with isolate recovery rates on days 5 and 10. Even on day 15 this correlation continued to hold (Figs 7 to 9). Clinical severity estimated on day 2 was a significant predictor of subsequent disease severity and isolate recovery. Thus, corneas with severe disease on day 2 (grade 3 or more) showed a continuation of the same pattern on subsequent days. Mildly diseased corneas (grade 2 or less on day 2) followed a significantly different course, remaining clinically mild on days 5 and 10 and virtually resolving by day 15 (Fig 10) (data analyzed by Wilcoxon rank sum test). A similar pattern was observed with isolate recovery rates. Corneas that had developed severe clinical disease on day

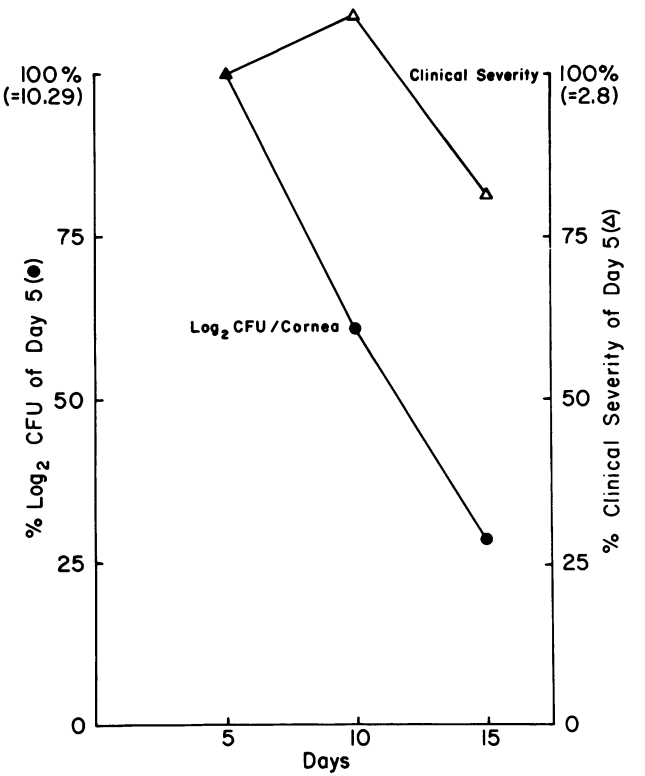


FIGURE 6
Course of experimental *Aspergillus fumigatus* keratitis over 15 days as measured by both clinical severity and quantitative isolate recovery. The curves are normalized to the 5th day.

2 (grade 3 or more) subsequently yielded numbers of organisms on days 5 and 10 that were higher than the numbers recovered from corneas with mild clinical disease on day 2 (grade 2 or less) (Fig 11). When analyzed by *t*-test, this difference was significant for day 5 ($P = 0.01$) and day 10 ($P < 0.05$).

II. NATURAL COURSE OF EXPERIMENTAL *CANDIDA ALBICANS* KERATITIS

The purpose of this study was to determine the natural course of *Candida albicans* keratitis in the rabbit as defined by isolate recovery rates following inoculation of the central corneal stroma.

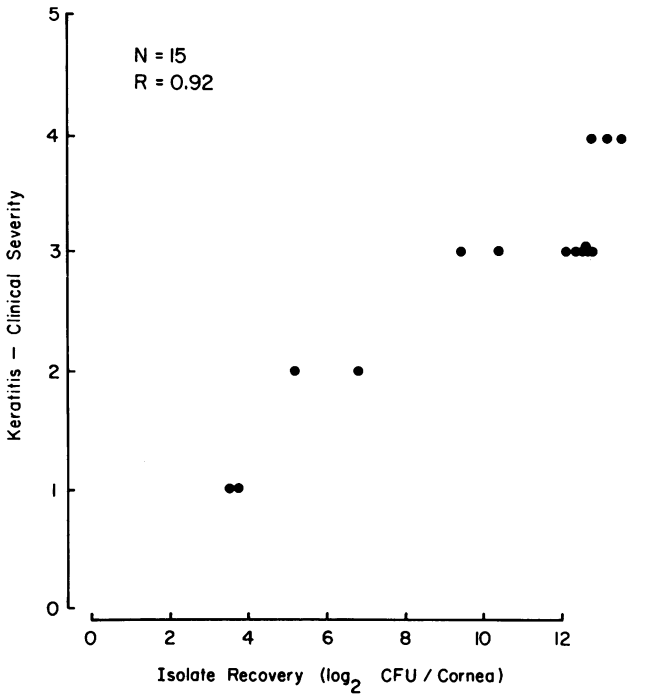


FIGURE 7
Correlation of severity of clinical disease with isolate recovery - day 5 of experimental *Aspergillus fumigatus* keratitis ($P = 0.92$).

MATERIALS AND METHODS

Inoculum

Candida albicans (strain VE102) was used in these experiments. This strain (formerly known as LV) causes a consistent and reproducible infection in the rabbit cornea following intrastromal inoculation.[17,27] For these studies the yeast was grown on commercially prepared plates of Sabouraud's dextrose agar (BBL, Cockeysville, MD) for 24 hours at 30°C. Blastoconidia were harvested and suspended in normal saline. The final concentration, adjusted so that a 1:10 dilution provided 25% T at 550 nm, was confirmed by plate counting.

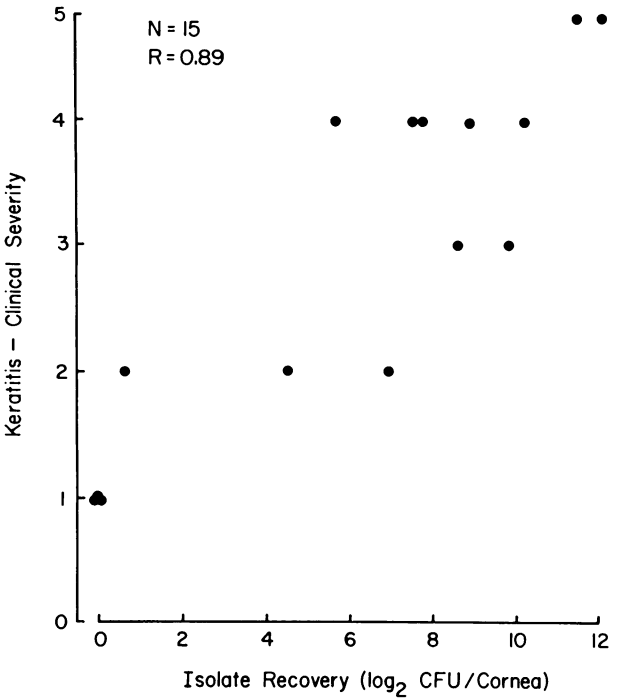


FIGURE 8
Correlation of severity of clinical disease with isolate recovery - day 10 of experimental *Aspergillus fumigatus* keratitis ($P = 0.89$).

Method of Inoculation

Dutch-belted rabbits (1.5 to 2.5 kg in weight) 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 100 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 yeast suspension were injected and the needle was removed.

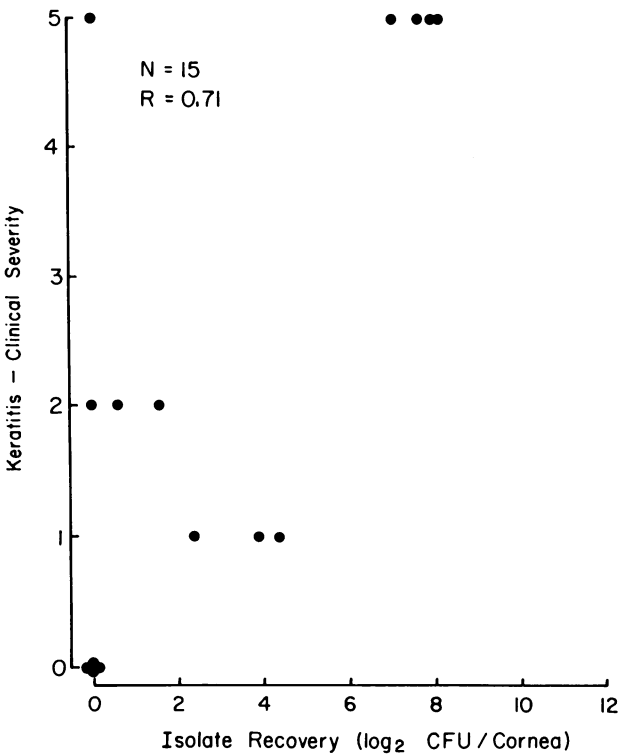


FIGURE 9
Correlation of severity of clinical disease with isolate recovery - day 15 of experimental *Aspergillus fumigatus* keratitis ($P = 0.71$).

Experimental Design

Immediately following inoculation, rabbits were randomly assigned to three groups. Group 1 was followed for 6 days, group 2 for 13 days, and group 3 for 17 days. At the conclusion of the designated period, all animals in the group were sacrificed and the corneas were processed for residual organisms by quantitative isolate recovery techniques.[17]

RESULTS

Six days following inoculation, disease as measured by the isolate recovery rate was very consistent among the eight animals in this group. At this

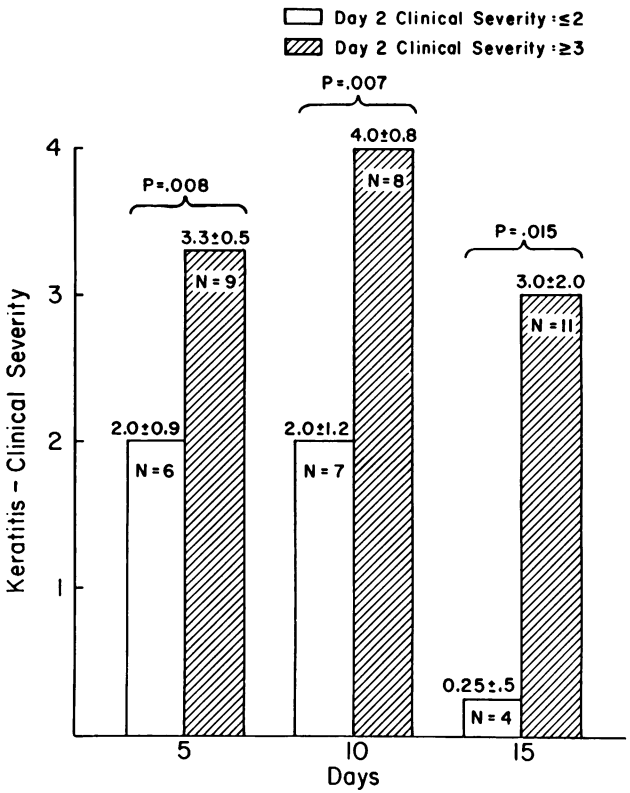


FIGURE 10
Experimental *Aspergillus fumigatus* keratitis. Comparison of subsequent clinical course in rabbits with clinically severe (grade 3 or above) or clinically mild disease (grade 2 or below) on day 2 following inoculation (mean ± standard deviation [SD]).

timepoint, a mean of 17.88 ($\log_2$) organisms were recovered with a standard error (SE) of 0.08. Seven days later the isolate recovery rate had declined to 14.88 $\log_2$ CFU while variance had increased (SE = 0.76). A further reduction in mean isolate recovery to 11.86 $\log_2$ CFU was apparent by the 17th day of infection. In addition, variance increased further as evidenced by the range in isolate recovery among the 12 animals (6.32 to 15.97 $\log_2$ CFU) and the corresponding increase in the SE to 0.92 (Table III).

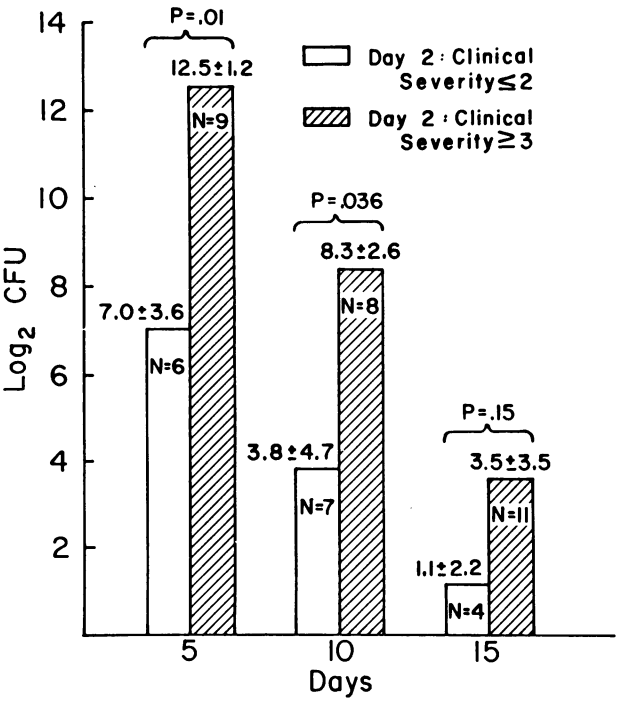


FIGURE 11
Experimental *Aspergillus fumigatus* keratitis. Comparison of subsequent isolate recovery rates in rabbits with clinically severe (grade 3 or above) or clinically mild disease (grade 2 or below) on day 2 following inoculation (mean ± SD).

III. PHARMACOLOGIC STUDIES

The purpose of these studies was to determine the uptake of fluconazole in the rabbit cornea following oral administration and to establish an optimal rate of administration. These data were necessary to determine the therapeutic approach in subsequent experiments involving *Candida albicans* and *Aspergillus fumigatus* species keratitis.

EXPERIMENT III-A. UPTAKE OF FLUCONAZOLE IN THE CORNEA FOLLOWING A SINGLE ORAL DOSE

TABLE III: QUANTITATIVE ISOLATE RECOVERY FROM RABBIT CORNEAS 6 TO 17 DAYS FOLLOWING INOCULATION WITH *Candida albicans*

CFU OF *Candida albicans* (log_2)					
DAY 6		DAY 13		DAY 17	
CORNEA		CORNEA		CORNEA	
1	18.02	9	16.36	17	15.97
2	17.69	10	13.10	18	10.80
3	17.73	11	11.93	19	11.96
4	17.92	12	12.00	20	8.32
5	18.10	13	16.07	21	15.46
6	17.75	14	16.50	22	7.71
7	18.26	15	16.88	23	14.76
8	17.61	16	16.19	24	14.32
				25	13.26
				26	6.32
				27	10.33
				28	13.14
Mean ± SEM	17.88 (0.08)		14.88 (0.76)		11.86 (0.92)

MATERIALS AND METHODS

Animals

Dutch-belted rabbits, 1.5 to 2.5 kg in weight were used.

Drug

Fluconazole powder was obtained as a gift from Pfizer Central Research in Groton, CT. For administration to the animals, gelatin capsules containing the powder were prepared so that each animal received a dose calculated according to its weight.

Drug Administration and Preparation for Assay

The rabbits were fed a single capsule of fluconazole containing either 10 mg/kg or 20 mg/kg. Four and eight hours later, groups of three animals receiving each dosage were sacrificed. Both eyes in each animal were immediately enucleated. The corneas were excised at the limbus, placed in cryotubes and frozen to −70°C. Together with serum samples collected from each animal immediately prior to sacrifice, these specimens were transported on dry ice to Pfizer Central Research for assay.

Drug Assay

Drug levels in the cornea were determined by high pressure liquid chromatography (HPLC) using the method described by Foulds and associates.[40] For the corneal tissue, this technique was modified as follows:

Each sample of the cornea was added to nine times its weight in water and homogenized using a Ultra-Turrax Tissumizer. One gram of the homogenate containing 100 mg of tissue was then extracted for assay. Standard curves were prepared from control tissues from undosed animals. Prior to drug assay, equivalent samples of control corneal tissue were examined to be sure there were no background peaks with retention time similar to those of the drug.

RESULTS

Fluconazole readily penetrated the corneas of these rabbits. Levels following a dose of 20 mg/kg were found to be almost double those achieved with the lower dose (10 mg/kg) (Table IV).

The amount of drug in the cornea was related to the serum level. At both dosages there was only a minor decline in corneal concentration during the 8-hour period. Corneal penetration of the drug (concentration in corneal tissues/concentration in serum) was high (Table V).

EXPERIMENT III-B. THE EFFECT OF RATE OF ADMINISTRATION ON UPTAKE OF FLUCONAZOLE IN THE CORNEA

MATERIALS AND METHODS

Animals

Dutch-belted rabbits 1.5 to 2.5 kg in weight were used. In order to study the influence of inflammation on corneal uptake, the right eye of each animal was inoculated with *Candida albicans* blastoconidia (VE102 strain) 24 hours prior to beginning drug administration. This technique has been shown to produce a rapid fungal invasion of the cornea.[27]

Drug Administration and Preparation for Assay

Fluconazole, in a dose of 30 mg/kg/day in gelatin capsules, was administered by mouth either as a single or a 12 hourly divided dose. Groups of three animals on each dose schedule were treated for periods of 1, 3 or 5 days. Animals receiving a single daily dose were sacrificed with T-61 euthanasia solution 24 hours after the last dose, while those on the 12 hourly dose schedule were sacrificed 12 hours after the last dose.

Drug Assay

The corneas were excised at the limbus and, together with serum samples collected immediately prior to sacrifice, were frozen in cryotubes for assay by HPLC.

TABLE IV: FLUCONAZOLE LEVELS IN CORNEA AND SERUM AFTER A SINGLE ORAL DOSE

EYES	NO.	DOSE (mg/kg)	TIME (hr)	MEAN CONCENTRATION (μg/ml or μg/gm) SERUM	CORNEA
4	2	10	4	3.1 ± 1.1	4.2 ± 1.0
6	3	10	8	2.2 ± 0.3	2.6 ± 0.5
6	3	20	4	6.5 ± 0.3	7.4 ± 0.3
6	3	20	8	4.9 ± 0.9	5.0 ± 0.5

TABLE V: CORNEAL PENETRATION OF FLUCONAZOLE FOLLOWING A SINGLE ORAL DOSE

EYES	NO.	DOSE (mg/kg)	TIME (hr)	MEAN RATIO (cornea/serum)
4	2	19	4	1.98 ± 0.37
6	3	10	8	2.60 ± 0.29
6	3	20	4	2.09 ± 0.32
6	3	20	8	2.47 ± 0.03

RESULTS

Fluconazole was present in all corneal and serum samples at all sampling times for the period of the study. There was no difference in corneal uptake between inflamed and normal corneas. Therefore, the results from both groups were pooled. The corneal concentration correlated strongly with that of serum ($R = 0.89$) (Fig 12). After 24 hours treatment, there was no significant difference in serum and corneal concentration in animals that received a single or divided dose (Fig 13). However, fluconazole appeared to accumulate in the corneas of animals treated by divided dosage over the 3 and 5 day periods, whereas the corneal concentrations did not change in those receiving a single daily dose. There was also a slow rise in the cornea to serum ratio over the 5-day period for the animals treated by divided dosage.

IV. THERAPEUTIC STUDIES WITH ORAL FLUCONAZOLE IN EXPERIMENTAL *CANDIDA ALBICANS* KERATITIS

The purpose of these studies was to evaluate efficacy of oral fluconazole in the treatment of *Candida albicans* keratitis.

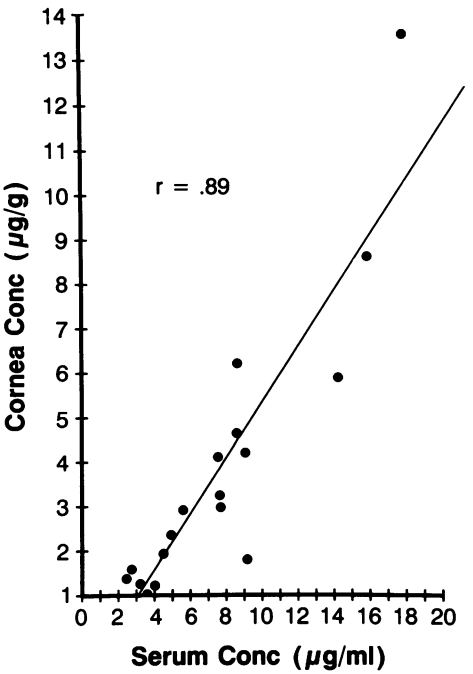


FIGURE 12
Correlation between serum and corneal concentrations of fluconazole after oral administration of 35 mg/kg/day.

MATERIALS AND METHODS

Candida albicans (strain VE102) was used in these experiments. The methods for preparation of the inoculum and inoculation of rabbit corneas are described in Part II of these studies.

Drug Administration

Gelatin capsules containing fluconazole were prepared so that each animal received a dose according to its weight. Capsules were administered orally at 12-hour intervals for periods determined by the particular treatment protocol. All animals in this and subsequent experiments receiving fluconazole orally were observed for the development of drug-related side effects.

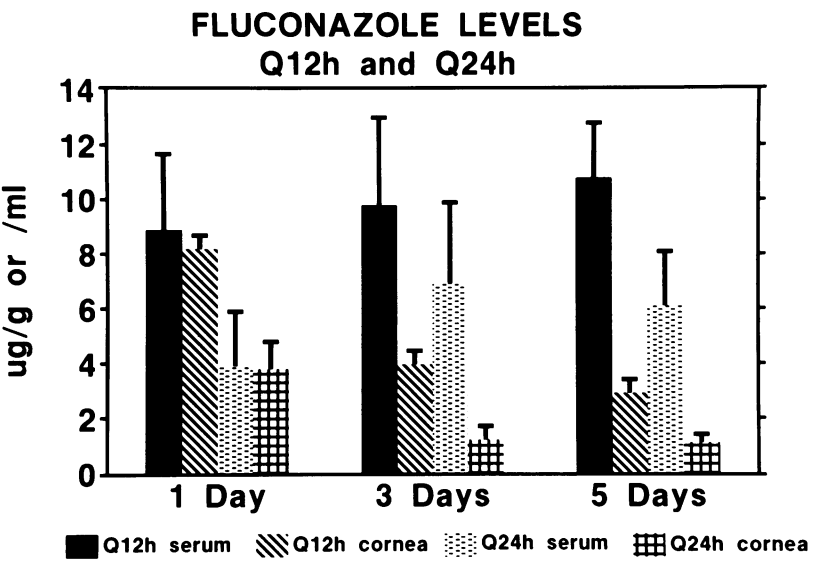


FIGURE 13
Fluconazole levels in serum and cornea following 1, 4 and 5 days oral administration in rabbits.

Quantitative Culture Technique

At the designated time animals were sacrificed and the number of CFU recovered from each cornea was estimated using a standardized isolate recovery technique.[17]

Statistical Analysis

The effect of drug treatment was estimated by subtracting the mean of the $\log_2$ CFU of treated animals from control animals yielding a difference termed Δ. An estimate of the SE of Δ was calculated and the hypothesis that Δ was significantly different from 0 was tested using analysis of variance techniques.[24,45] An effect was considered significant if the significance probability was ≤ 0.05 and marginally significant if > 0.05 but ≤ 0.10.

EXPERIMENT IV-A. EFFECT OF PREINOCULATION TREATMENT ON ISOLATE RECOVERY IN *CANDIDA ALBICANS* KERATITIS

Experimental Design

Twelve healthy Dutch-belted rabbits (1.5 to 2.5 kg) were randomly allo-

cated to two equal groups. One group served as untreated controls. The other group received oral fluconazole in a dose of 17.5 mg/kg at 12-hour intervals for three doses. Four hours after the last dose, *Candida albicans* infection was established in the right eye of each animal from both groups. Twenty-four hours after inoculation all animals were sacrificed and the number of CFU recovered from each cornea was estimated using quantitative isolate recovery techniques.

RESULTS

Pretreatment with oral fluconazole without further treatment resulted in a statistically significant reduction ($P = 0.01$) in the number of CFU isolated 24 hours later as compared to untreated controls (Fig 14).

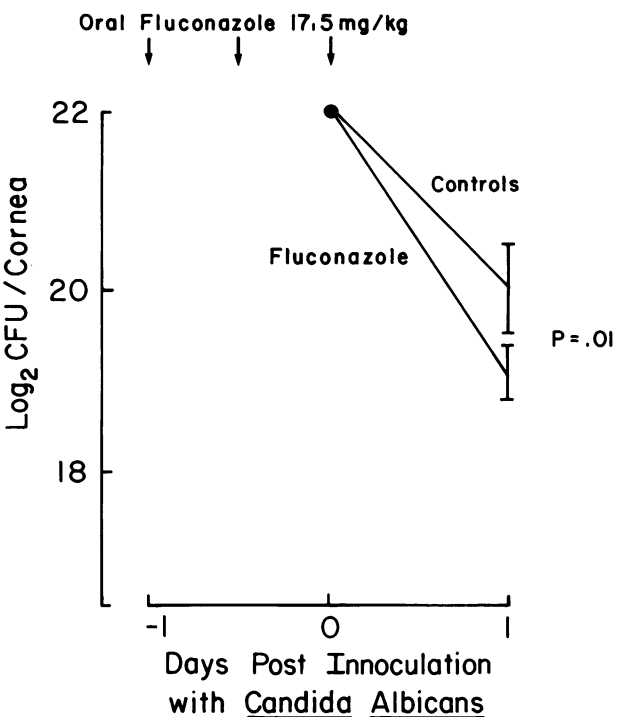


FIGURE 14

Experimental *Candida albicans* keratitis. Response to pretreatment with 3 doses of fluconazole 17.5 mg/kg 12 hours apart beginning 24 hours before inoculation, as measured by quantitative isolate recovery; 6 rabbits in each group (mean ± SD).

EXPERIMENT IV-B. THE EFFICACY OF COMBINED PRE- AND POSTINOCULATION TREATMENT COMPARED TO POSTINOCULATION TREATMENT ONLY

Experimental Design

Eighteen healthy Dutch-belted rabbits (1.5 to 2.5 kg) were randomly allocated to three groups of six animals. *Candida albicans* keratitis was established in the right eye of each animal. One group served as untreated controls. A second group received oral fluconazole 17.5 mg/kg every 12 hours for a total of 14 doses, including three given over the 24 hours prior to inoculation and one 12 hours following inoculation. The third group of animals received 10 doses of the drug, (17.5 mg/kg) every 12 hours for a 5-day period beginning 24 hours after corneal inoculation. Eighteen hours after the last dose all animals in each group were sacrificed and the corneas were processed for quantitative isolate recovery.

RESULTS

The number of CFU recovered was reduced in each treatment group compared to the control animals ($P = 0.05$ delayed treatment, $P = 0.08$ pre- and postinoculation treatment). There was, however, no significant difference between the effects of these two treatments. One animal died, reducing the number of corneas in the combined pre- and posttreatment group to five (Fig 15).

V. THERAPEUTIC STUDIES WITH ORAL FLUCONAZOLE IN EXPERIMENTAL *ASPERGILLUS FUMIGATUS* KERATITIS

The purpose of these experiments was to determine the efficacy of oral fluconazole therapy for experimental *Aspergillus* keratitis as determined by the clinical course of the disease and isolate recovery rates at the conclusion of the treatment period. On the basis of the experiments in Part I, a treatment period of 5 days was selected.

MATERIALS AND METHODS

Inoculum

Aspergillus fumigatus (strain VE148) was used for these studies. Prior to each experiment, the strain was inoculated on a commercially prepared plate of Sabouraud's dextrose agar and grown to confluence for 3 to 4 days. Spores harvested by loop were suspended in 15 ml of normal saline containing 50 μl of Tween-80. The suspension was diluted to 95% T at 550 nm on the spectrophotometer and the final concentration was verified by plate counting.

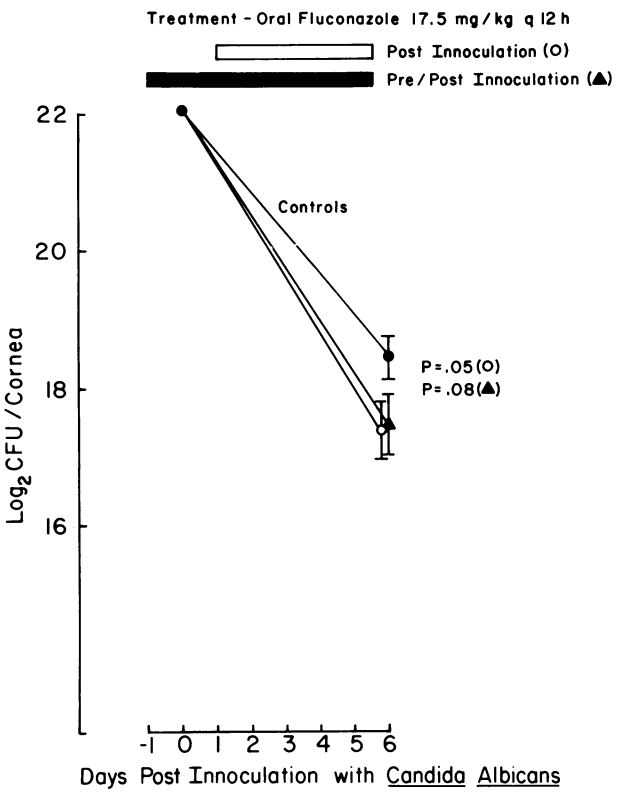


FIGURE 15

Efficacy of combined pre- and postinoculation treatment compared to postinoculation treatment in experimental *Candida* keratitis as measured by quantitative isolate recovery. Oral fluconazole 17.5 mg/kg was given every 12 hours; 6 rabbits in each group (mean ± SD).

Method of Inoculation and Evaluation of Response

The techniques as described in Part I were used. Statistical analysis of clinical scores was performed by the Wilcoxon rank sum test. The same approach as that outlined in the previous series of experiments with *Candida albicans* was employed for statistical analysis of isolate recovery data.

EXPERIMENT V-A. EFFICACY OF COMBINED PRE- AND POSTINOCULATION TREATMENT IN *ASPERGILLUS* KERATITIS

Experimental Design

Eight healthy Dutch-belted rabbits (1.5 to 2.5 kg) were administered oral fluconazole (25 mg/kg) every 12 hours for three doses. Four hours after the third dose, the right cornea of each of these animals and of eight untreated controls was infected with *Aspergillus fumigatus*. Oral fluconazole was continued in the treated group for five more days. On the 5th day, a clinical score was allocated to treated and control corneas by an observer masked as to the treatment group. Eighteen hours after the last dose, all animals were sacrificed and quantitative isolate recovery techniques were used to estimate the number of CFU isolated from the corneas.

RESULTS

Only two animals in the treatment group exhibited severe disease clinically (Table VI). The cornea of one treated animal healed completely; the remainder of treated animals had evidence of mild infection. All untreated control animals exhibited severe clinical disease. This effect of treatment was statistically significant ($P < 0.05$). Isolate recovery rates in the treated animals were significantly reduced compared to controls ($P = 0.05$) (Fig 16).

TABLE VI: REDUCTION IN CLINICAL SEVERITY FOLLOWING COMBINED PRE- AND POSTINOCULATION TREATMENT WITH ORAL FLUCONAZOLE

FLUCONAZOLE TREATMENT		UNTREATED CONTROLS	
RABBIT NO.	SEVERITY SCORE	RABBIT NO.	SEVERITY SCORE
922	1	909	3
916	2	915	3
917	0	914	4
923	2	908	3
913	1	918	3
921	3	910	4
906	4	920	4
858	1	912	3

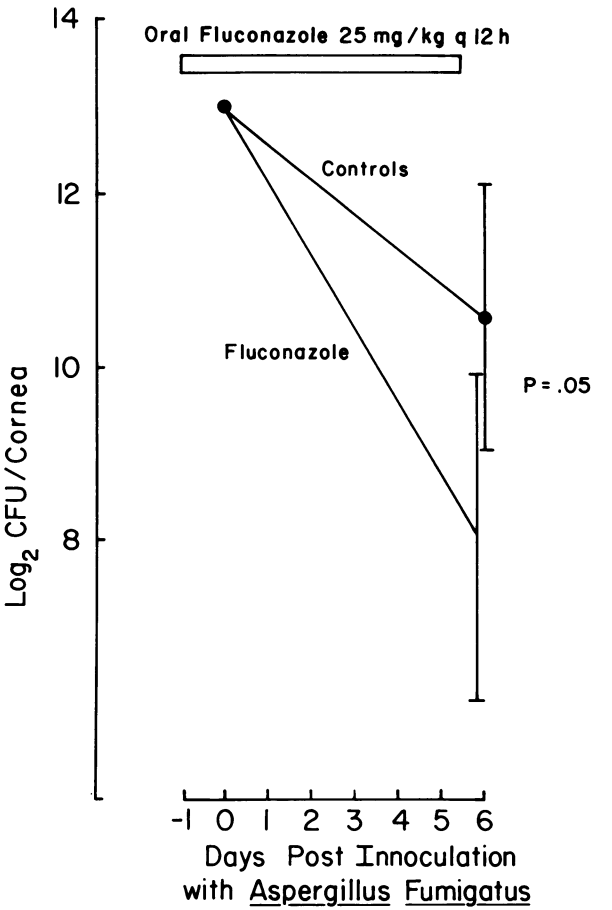


FIGURE 16
Efficacy of combined pre- and postinoculation treatment with oral fluconazole (25 mg/kg 12 hourly) in experimental *Aspergillus fumigatus* keratitis as measured by quantitative isolate recovery; nine rabbits in each group (mean ± SD).

EXPERIMENT V-B. EFFICACY OF COMBINED PRE- AND POSTINOCULATION TREATMENT COMPARED TO TREATMENT POSTINOCULATION ONLY

Experimental Design

Thirty-six Dutch-belted rabbits (1.5 to 2.5 kg) were randomly allocated to three groups of 12 animals each. Three doses of oral fluconazole (25 mg/kg every 12 hours) were administered to the first group. Four hours after the

third dose, the right corneas of these animals and of the animals in the other two groups were inoculated with *Aspergillus fumigatus*. Oral fluconazole treatment was continued in the first group until the sixth postinoculation day. Treatment in the second group was begun 24 hours after inoculation and continued for 5 days. The third group served as untreated controls. On the last day of treatment, all corneas were examined and allocated a clinical score by an observer masked as to the treatment group. Eighteen hours after the last dose the animals were sacrificed and the corneas were processed for quantitative isolate recovery.

RESULTS

On the last day of treatment, the mean clinical score for both treatment groups was less than that of untreated control animals. This was highly significant ($P < 0.001$) for the group of animals undergoing combined pre- and postinoculation treatment but was significant only at the $P < 0.1$ level for the postinoculation group (Fig 17). Isolate recovery rates were also reduced in the treated animals. Significantly fewer organisms were recovered from corneas undergoing pre/postinoculation treatment than untreated controls ($P \leq 0.05$). Fewer organisms were also recovered in the postinoculation treatment group, but this was significant only at the $P < 0.1$ level (Fig 18).

Side Effects of Fluconazole

Systemically administered fluconazole appeared to be well tolerated by all the animals in these short-term experiments. Feeding remained normal, observed activity was normal and weight was maintained. The experiments were not designed to evaluate side effects of delayed onset or the consequences of a more prolonged period of administration.

DISCUSSION

These studies of the efficacy of systemic therapy for experimental keratomycosis proceeded in three stages. First it was necessary to identify suitable animal models of infection. Next, corneal penetration of oral fluconazole was studied to develop an optimal therapeutic regimen. Finally, the efficacy of oral fluconazole in the treatment of keratomycosis due to *Candida albicans* and *Aspergillus fumigatus* was evaluated.

ANIMAL MODEL DEVELOPMENT

Two separate models were used. For the experiments involving *Candida albicans*, a model of deep corneal infection previously developed to study

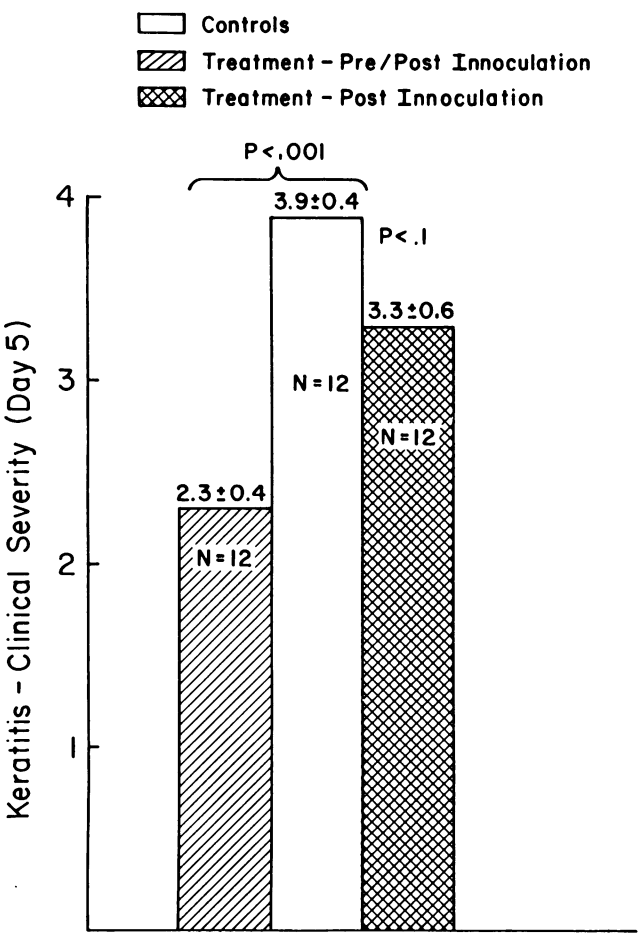


FIGURE 17
Clinical response after oral fluconazole therapy (25 mg/kg 12 hourly) in experimental *Aspergillus fumigatus* keratitis; combined pre- and postinoculation treatment compared to untreated controls and postinoculation treatment only (mean ± SD).

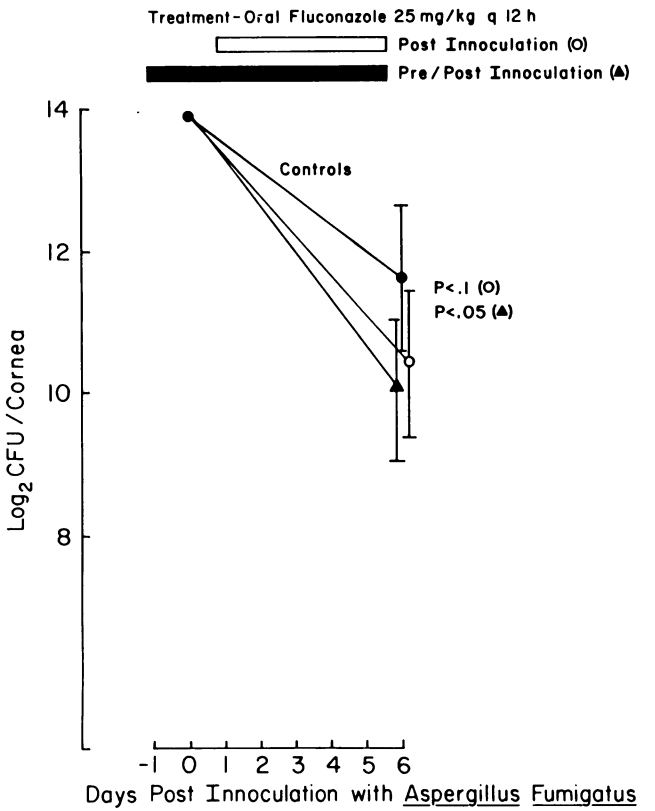


FIGURE 18
Efficacy opf combined pre- and postinoculation treatment with fluconazole (25 mg/kg at 12-hour intervals) compared to postinoculation treatment in an animal model of *Aspergillus fumigatus* keratitis as measured by quantitative isolate recovery; 12 rabbits in each group (mean ± SD).

topical therapy appeared suitable.[17] In this model, invasive disease is established within 24 hours of inoculation.[27] Without modification of the host animal with corticosteroid, isolate recovery rate are highly consistent for at least 6 days.[17] The present study confirmed these findings and showed that the number of residual organisms was still high 1 week later though variance within the group was increasing moderately as the infection began to resolve in individual animals (Table III).

Previous investigators have experienced difficulties in infecting animal corneas with *Aspergillus* without the use of steroid,[18,28] so it was essential

that a new model be defined and characterized before treatment studies were begun. In view of the uncertain effects of steroid on the host, fungal growth, and activity of the antifungal agents, an important criterion for a successful animal model was the development of an infection of sufficient intensity and duration without recourse to steroid.

Several strains of *Aspergillus fumigatus* were evaluated before one was found which produced a gradually progressive disease in most animals. Without host modification, infection with this strain appears to persist about 10 days. Beyond this time, very few viable organisms are present in the cornea.

Mycological confirmation of the model was a major goal of this study. It could be sought in one of several ways. The cornea could be cultured periodically in the course of the experiment. Groups of animals could then be compared on the basis of the culture results. However, the poor sensitivity of this technique is such that false negative cultures would be likely to confuse interpretation of the results. A second approach is to culture the whole cornea at the conclusion of the treatment period. While probably more sensitive than the previous method in identifying corneas with residual organisms, graduated responses are not measured. Finally, a quantitative approach could be employed in which the actual number of viable organisms in the cornea is determined by plate and counting methods. With the appropriate statistical analysis, quite small differences, such as might occur in short term models, can be detected provided the variance is low.[30]

Quantitative methods are appropriately used for infections involving bacteria since every CFU represents at least one viable bacterium.[46-48] Their validity in yeast infections has been established.[16,17,25,29] The present study, using a standardized method of tissue fragmentation, supports the validity of this technique in infections with molds also. Very little variation in isolate recovery rates was seen among groups of corneas infected with a standardized inoculum of *Aspergillus fumigatus*. Altering the grinding time also had little effect. Even when it was tripled, the impact on recovery rates from samples of individual corneas appeared negligible (Tables I and II).

The system for scoring the clinical disease, developed during pilot studies on untreated animals, was empirical at its inception. Division of the disease into five stages of increasing severity. Using this grading system, it was apparent that although some animals appeared resistant, the majority developed a progressive infection. Animals resistant to infection could be detected on the second postinoculation day by the failure to develop severe corneal inflammation. Disease in these corneas (grade 2 or

less) usually remained clinically mild and subsequent isolate recovery rates were low. On the other hand, corneas with severe disease on the second day (grade of 3 or more) continued to exhibit severe inflammation. Isolate recovery rates in these corneas were also significantly higher than in the clinically mild group. Although not part of the design of the present study, the predictive value of the clinical score on day 2 is a valuable characteristic of the model and could be used to reduce variance. By selecting animals with a severity score of grade 3 or higher on day 2, a group with relatively uniform disease could be selected for treatment studies.

The correlation between isolate recovery rates and severity of disease on the days that both observations were made, indicates a relationship between the clinical observations on these days and the number of viable fungal elements present in the cornea. This correlation was strongest on days 5 and 10. However, despite this clear relationship on individual days, the two parameters diverged steadily during the 15 days of observation (Fig 6), so that progressively fewer viable organisms were necessary to maintain a given clinical score. The impact of a developing host inflammatory response on the clinical score best explains this dissociation. As noted previously, clinical scores reflect primarily the intensity of the host response. The results of this study indicate that they measure mycologic events only indirectly, at least in this short-term model. For this reason, quantitative isolate recovery methods appear to be the more valid means of evaluating antifungal activity in this model. Clinical observations, when interpreted with the mycologic data, provide useful confirmatory information but are of uncertain value when used alone.

DRUG PENETRATION STUDIES

The corneal levels of fluconazole in rabbits receiving the drug orally confirmed the previous observation of excellent corneal penetration following intravenous administration.[35] While caution should be exercised in extrapolating information from experiments involving rabbits to man, these data confirm experience with other animal systems and point to the generally excellent tissue levels achieved by oral administration.[14,40]

As a consequence of the design of the 5-day drug penetration study, trough rather than peak levels in the cornea were measured. As such they indicate that a twice daily rate of administration is likely to be more effective in rabbits than a single daily dose. For this reason, the animals in the therapeutic studies were treated every 12 hours. However, it is important to note that the half-life in rabbits is considerably shorter than in man (13 hours versus 24 to 30 hours).[14] Thus, in man it is quite possible

that a once-a-day dose would be optimal. More important was the demonstration of an apparent accumulation of drug over the 5-day period. Whether further accumulation occurs with continued treatment is presently unknown. In man, the serum concentration reaches a steady state after approximately 5 days of once-a-day dosing.[49] Although the validity of extrapolating serum data to tissue is unknown, drug accumulation in tissue is an important issue with implications that affect both therapy and toxicity, particularly since prolonged administration may be required.

ORAL FLUCONAZOLE IN THE TREATMENT OF KERATOMYCOSES

Fluconazole, like other azole compounds, is fungistatic at the concentrations likely to be attained in the cornea by oral administration.[50] Its mechanism of action appears to be an active inhibition of hyphal growth.[51] With continued exposure to the drug, hyphal formation ceases and the organism gradually dies. The period required for complete elimination of an established infection is likely to be prolonged, as shown by the duration of treatment in other experimental infections.[41-43,52] However, a progressive reduction in the number of viable organisms recovered should occur if the organism is susceptible.

An analogy is seen in the responses to topical natamycin when two different treatment protocols were used. These experiments were performed in the same model of *Candida* keratitis as that used in this study.[27] In one protocol, treatment was begun immediately following inoculation when the yeast was still in the spore phase. In the other, treatment was delayed 24 hours to allow transformation to the hyphal phase. The therapeutic effect of natamycin demonstrated with immediate treatment, as measured by the number of organisms isolated from the cornea, was much reduced when the infection was allowed to become established prior to treatment. While this finding might be interpreted to indicate a low level of efficacy for natamycin, clinical experience shows the agent to be effective in established yeast infections when the organism is in a hyphal growth phase even though prolonged treatment may be required.[3,4] Thus, the reduced but still significant effect noted when invasive disease is established may be a manifestation of the predominantly fungistatic action of natamycin. It has to be remembered that these studies were performed in models that are necessarily short-term, whereas treatment of clinical disease is usually prolonged even with highly efficacious agents. The difficulty lies in extrapolating experimental findings to the treatment of clinical disease. However, different responses at different stages of the experimental infection appear to be valid indicators of efficacy even though they are not directly comparable.

Given the limits of these two models, it was essential to design treatment strategies that would offer the best chance of demonstrating a therapeutic effect. A treatment period within the 10-day life of these models was unlikely to be of sufficient length to expect a clinical and mycologic cure of an established infection. A compromise, therefore, had to be made regarding the time therapy was initiated. While delaying treatment for two days might have allowed stratification of animals into groups with comparable disease, this approach would have severely limited the period available for treatment.

Another approach is to examine the protective effect afforded by the drug when administered for a period prior to inoculation. The evidence of drug accumulation in the cornea after 1 day of treatment suggested that a pretreatment period of 24 hours might be sufficient to demonstrate an effect. Since the inoculum in each model is composed of organisms in the spore or blastoconidial phase, it was reasoned that if the organism was susceptible, transformation to the hyphal phase would be inhibited and a therapeutic effect should be detectable even though organism was susceptible, transformation to the hyphal phase would be inhibited and a therapeutic effect should be detectable even though organism death may be incomplete. The effect would presumably be amplified if treatment was continued following inoculation. A reduction in clinical severity should also be apparent if fungal invasion is inhibited by the drug. This strategy appears to provide the best opportunity to demonstrate a therapeutic effect.

In the initial experiment with the *Candida albicans* infection (Fig 14) pretreatment did exert a protective effect with just three doses of the drug. The reduction in CFU was significant ($P = 0.01$). In subsequent experiments with both models in which treatment was continued for 5 days after inoculation, there was also a consistent reduction in the number of viable organisms recovered. These animals also had significantly milder clinical disease, possibly due to a reduction in invasive disease as a result of hyphal inhibition.

As might be anticipated, oral fluconazole fared less well in these models when treatment was withheld until invasive disease had become established. Yet again, the same trend towards reduced organism counts was apparent. In contrast to the pretreatment group, however, the reduction in clinical disease was not significant although an improvement was noted in many of the animals. A possible explanation for this disparity is the heightened host inflammatory response to the invasive fungal disease already established before treatment was begun. The finding also emphasizes a lack of specificity in the clinical scoring system and is further

evidence against the use of nonparametric measures without the support of objective mycologic data.

These studies provide evidence for the efficacy of fluconazole when given orally for experimental keratomycosis due to *Candida albicans* and *Aspergillus fumigatus*. Savani and associates,[35] in the only other ocular study of systemic fluconazole, also found the agent to be efficacious in the treatment of experimental *Candida* endophthalmitis when given intravenously.

In the absence of comparable data with other oral agents, an assessment of relative efficacy in experimental *Candida albicans* keratitis cannot yet be determined. Some comparison with topical therapy is possible since experiments with the same infecting strain have been reported.[17,25] In a model of infection in the superficial cornea, topical miconazole or flucytosine, given immediately following inoculation, appeared to provide protection of the same order of magnitude as that seen with the present study. Topical ketoconazole was ineffective. However, topical amphotericin B virtually abolished the infection. Natamycin had an intermediate but highly significant effect.[25] When the same strain was used in a model identical to that employed in this study, only amphotericin B and natamycin were efficacious.[17] Miconazole, ketoconazole and flucytosine did not significantly reduce the number of CFU isolated despite high rates of administration. Thus, at least for the *Candida albicans* infection, oral fluconazole appears to be superior to topical imidazoles and flucytosine. Clearly though, such conclusions are tentative in the absence of appropriately designed comparative studies. A comparison with other oral agents for experimental *Aspergillus fumigatus* keratitis is not available since the results were inconclusive with ketoconazole, the only other agent studied to date.[18] However, the efficacy of topical natamycin appeared superior to anything observed with oral fluconazole.[18]

The present study did not investigate the value of oral fluconazole when given in combination with topical antifungal agents though clearly this is an important role oral therapy may fill and is deserving of investigation. Another important step should be the evaluation of other experimental agents in relation to fluconazole in these models. One of these, itraconazole, when given intravenously has already been shown to be efficacious in the treatment of experimental *Candida albicans* endophthalmitis.[35] Studies with experimental *Aspergillus* in other organ systems also reveals a promising therapeutic effect.[53]

Animal models of infection are important in the preliminary evaluation of in vivo antifungal activity. However, the relatively short course of disease in animal corneas can frustrate this assessment particularly if the

agent is fungistatic and requires a lengthy period of administration. By carefully characterizing the model, this obstacle can be minimized, as these studies have attempted to show. Knowledge of the limits of the model make it feasible to design appropriate treatment protocols and to select methods for evaluation that are sufficiently sensitive for the task. It may then be possible in a carefully characterized model to introduce the variable of host modification by steroid administration or other means with the goal of prolonging the period available for treatment. However, before such a model can be considered to be adequately representative of human disease the effect of the modification on fungal growth, drug action and the disease itself will need to be assessed. Although this may seem a formidable task, observations of the type described in these experiments provide a useful starting point.

SUMMARY

Fluconazole, an experimental azole antifungal agent with good tissue penetration following oral administration, offers the possibility of a new approach to the treatment of keratomycosis. Its efficacy as an orally administered agent was investigated in two models of experimental fungal infection in Dutch-belted rabbits. The study proceeded in three stages. In the first, a model of keratitis due to *Aspergillus fumigatus* was developed, the suitability of quantitative isolate recovery techniques for the evaluation of the disease caused by this organism was confirmed, and the correlation between the severity of clinical disease scored nonparametrically and the isolate recovery rate was established. The model was found to be most useful for study during the first 5 days of infection. The natural course of experimental *Candida alibcans* keratitis was evaluated and, on the basis of quantitative isolate recovery techniques, this model was found to be appropriate for studies lasting up to 1 week.

In the second stage, corneal uptake following oral administration of fluconazole was studied in Dutch-belted rabbits. The drug was found to readily penetrate the cornea in amounts that correlated with serum levels ($R = 0.89$). Eight hours following a single 20 mg/kg dose, the corneal level was 7.4 mg/gm, almost double the amount when a 10 mg/kg dose was administered. When given in a twice daily divided dose, fluconazole accumulated steadily in the corneas over a period of 5 days. The presence of inflammation induced by fungal infection did not influence corneal uptake.

In the final stage, the efficacy of orally administered fluconazole in the treatment of keratomycosis was evaluated. Overall, a significant therapeu-

tic effect was observed with both infections. Treatment of the animals with oral fluconazole for 1 day prior to inoculation with *Candida albicans* led to a significant decrease in isolate recovery 1 day later ($P = 0.01$). However, when treatment was continued for 5 days following inoculation, no additive effect of pretreatment was noted. Pretreatment for 1 day followed by 5 days postinoculation treatment led to a significant decrease in clinical disease ($P < 0.05$) and isolate recovery ($P = 0.05$). A beneficial effect of pretreatment compared to treatment begun 1 day postinoculation, as measured by a reduction in clinical severity and isolate recovery, was also noted. On the basis of these short-term therapeutic studies and the excellent corneal penetration of fluconazole, further investigation of oral therapy of keratomycoses appears warranted.

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