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Evaluation of an Experimental Animal Model for Testing Antifungal Substances

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Accumulated evidence indicates that infection by fungi capable of causing systemic disease usually results in a relatively strong acquired resistance. The working hypothesis for this study was that an antifungal substance, even one with only slight fungistatic activity, could be an effective chemotherapeutic agent by arresting progression of the infection until acquired resistance became effective. The present study involved establishing and evaluating an experimental animal model (coccidioidomycosis in mice) which could be used to test this hypothesis. This model was reasonably similar to the natural disease. Results with infected nontreated animals indicated that the plot of mortality frequency against survival time did not follow a normal distribution curve. Thus, nonparametric procedures were used for evaluation. Use of this model with an established antibiotic (amphotericin B), with a crude preparation of a new antibiotic (CB-310), and with synthetic organoselenium compounds demonstrated that even low levels of antifungal activity could be detected. The model should be useful not only to test the original hypothesis but also to screen antifungal substances for their potential as chemotherapeutic agents.

The search for antifungal antibiotics has been attended with few successes. This is particularly true in the case of systemic mycoses, for which only amphotericin B has proven to be clinically useful. Even amphotericin B has limitations because of toxicity and fungistatic rather than fungicidal activity. Although fungicidal activity would be a desirable property, it may not be a necessary requirement. There is convincing evidence that convalescence from naturally acquired systemic mycoses is associated with immunity to reinfection (4), and, therefore, a fungistatic compound could be an effective chemotherapeutic agent if it slowed or arrested the progression of the disease until protective immunity developed. A series of investigations have been initiated with the ultimate objective of bringing experimental studies of potential chemotherapeutic agents to clinical trial. A most necessary step toward this objective is establishing an animal model in which the outcome of the infection can be predicted with a measurable level of confidence. The present report deals with this initial objective.

The experimental animal model must satisfy several conditions. Since either a fungistatic or a fungicidal agent may be effective, the experimental design for evaluating the efficacy of the drug must include the potential for detecting and measuring the lesser activity (i.e., fungistatic) as well as the stronger (i.e., fungicidal). This consideration would dictate that the infecting dose of fungus cells must not be so great as to produce an overwhelming infection which might mask a low level of control of the infection, and that the experimental infection must not be so slight that the effect of the drug cannot be distinguished from the naturally developing immune response. In addition, the course of the experimental animal disease should approximate that of the naturally occurring systemic mycosis. In the latter, infection is followed by an incubation period of 1 week or longer before the appearance of clinical symptoms. This leads either to convalescence and immunity or to progression of the disease with a chronic course of variable duration. The animal model should provide a similar pattern, i.e., infection, an incubation period of several days, and symptomatic disease. If the model is designed so that a measurable end point occurs after the onset of symptomatic disease and before the development of efficient immunity, it would be useful for detecting and evaluating compounds with even slight antifungal activity.

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Coccidioidomycosis in mice was chosen as the experimental animal model for several reasons. Immunity resulting from this infection is stronger and is documented better than that in other mycoses. In addition, Levine and his associates have demonstrated that mice have developed effective immunity against challenge infection approximately 30 days after vaccination (5, 6). During our past experience, we had established a model in which infection only occasionally resulted in death of animals before the 10th day postinfection but would kill 95 to 100% of the animals by the 30th day. These conditions satisfied to a reasonable degree those required for the experimental model as described above.

MATERIALS AND METHODS

A single strain of *Coccidioides immitis* (C-34) was used in the experiments reported. Cultures were grown on glucose-yeast extract-agar (1) at room temperature until extensive arthrospore formation had developed, usually in 1 to 2 months. Suspensions of arthrospores were harvested by layering minimal amounts of sterile saline over the agar surface, introducing a sterile Teflon-coated magnetic bar (0.8 mm diameter, 3.6 mm length), and stirring gently by placing the entire culture plate on a magnetic stirrer (8). Cell suspensions consisted almost entirely of single arthrospores with occasional short hyphal strands and unfragmented chains of two or three arthrospores. Visual arthrospore counts were made by hemocytometer, and the suspensions were adjusted to contain 2×10^5 arthrospores per ml. Viable counts were obtained by dilution, plating, and averaging the number of colonies on all countable duplicate plates. The range of viable counts for seven separate experiments was 0.80×10^5 to 2.08×10^5 spores per ml of the suspension. Since 0.5 ml of the cell suspension was injected, the mice received 0.40×10^5 to 1.04×10^5 viable spores. These differences did not significantly affect mortality frequency or survival times among the several experiments.

All animals were obtained from our colony of inbred dba mice. Only males of the same age (3 months) were used in each experiment. Mice were weighed just prior to infection, again at the initiation of treatment, and weekly thereafter. The averaged initial weight for all animals was 25.1 g with a range of 22.6 to 29.8 g and a standard deviation of ± 1.9 g. Randomly selected animals were infected by intraperitoneal injection with 0.5 ml of the standardized arthrospore suspension, and were then distributed, again at random, into the several groups used in each experiment. The number of mice per group in the several studies varied between 5 and 10, depending on the total number of animals available. Food and water were available ad libitum at all times.

The antifungal agents used in these studies were the antibiotics amphotericin B and CB-310, and several synthetic organoselenium compounds which had exhibited antifungal activity in experiments in vitro (*to be published*). (CB-310 is a new antibiotic supplied by A. Prieto, Calbiochem, San Diego, Calif. The organoselenium compounds were supplied by K. Schwarz, Veterans Administration Hospital, Long Beach, Calif.) The antifungal agents were dissolved in dimethyl sulfoxide (DMSO) in sufficient concentration so that the subsequent dilutions used for treating the mice contained a final concentration of 10% DMSO. Groups of infected animals as controls were treated with saline and with 10% DMSO on the same schedule as those receiving chemotherapy. Since there was no difference between these two control groups, the results for both have been pooled. Treatments were begun on the 5th day postinfection, since earlier studies had shown that the infection was established extraperitoneally by this time. Therapy was administered by daily intraperitoneal injection of 0.5 ml of drug dissolved in 10% DMSO for 5 days. Deaths were recorded in the morning every day of the week for 30 days, and survivors on day 30 were sacrificed. Since *C. immitis* arthrospores convert to characteristic endosporulating spherules in vivo, this was used as the criterion for determining that infection had been established. Animal tissues, particularly lung, liver, and spleen, were examined microscopically.

RESULTS

The principal question posed in these studies was whether the established experimental animal model of coccidioidomycosis would be appropriate for detecting and evaluating in vivo antifungal activity. The essential problem was to measure not only survival, but also a significant increase in survival time for treated animals compared to controls. Statistical methods are available for analyzing this type of problem. The first objective was to determine which of these methods was appropriate for evaluating the type of results generated by the experimental model. If the frequency of mortality among infected animals without treatment followed a normal distribution curve, then parametric statistics based on normality could be used; if not, then nonparametric statistical tests for significance were to be used. Plots of mortality frequency against time in models of chronic infectious disease usually reveal a skewed distribution (e.g., 12); therefore, the assumption that this characteristic is distributed normally within a given population of animals may or may not be justified, depending upon the extent to which the skewed distribution deviates from the normal. The first objective, therefore, was to determine whether survival times among infected nontreated mice in this experimental model were distributed normally.

The complete results for control animals from seven separate experiments are presented in Table 1, with the plot of the frequency distribution in Fig. 1. Only 2 of the 70 mice died before the 10th day, and 97% of the animals did not survive to the 30th day. Clinical evidence of the infection

TABLE 1. *Survival times for infected mice with no antifungal therapy*[a]

Day	Expt no.							Totals
	1	2	3	4	5	6	7	
<9	0	0	0	0	0	0	0	0
9	0	2	0	0	0	0	0	2
10	0	0	1	0	2	1	1	5
11	1	3	1	0	2	1	0	8
12	4	1	2	4	1	2	1	15
13	1	1	2	1	0	1	3	9
14	1	0	1	4	1	2	3	12
15	1	0	0	1	0	1	0	3
16	1	0	1	0	1	0	1	4
17	0	0	0	0	2	1	0	3
18	1	1	0	0	0	0	0	2
19	0	0	0	0	0	1	0	1
20	0	0	0	0	0	0	0	0
21	0	1	1	0	0	0	1	3
22	0	0	0	0	0	0	0	0
23	0	1	0	0	0	0	0	1
24-29	0	0	0	0	0	0	0	0
30[b]	0	0	1	0	1	0	0	2
Totals	10	10	10	10	10	10	10	70

[a] Average day of death (68 mice) = 13.56. Standard deviation = ±2.97.
[b] Survivors.

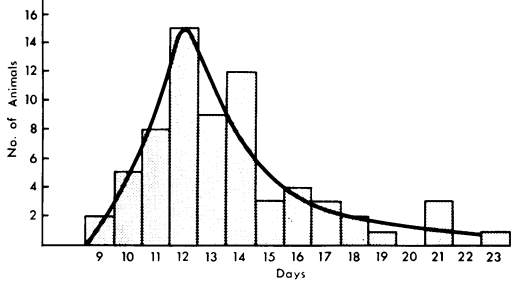

FIG. 1. *Distribution of survival times and mortality frequency for infected control mice dying in less than 30 days.*

(weight loss, lethargy, ruffled fur) was apparent in all mice by the end of the first postinfection week, but the two survivors appeared well and were gaining weight at the time of sacrifice on day 30. Endosporulating spherules were demonstrated in lesions within every animal, including the survivors. The curve illustrating the mortality frequency (Fig. 1) was skewed, and, therefore, the question of whether this distribution deviated significantly from a normal distribution had to be tested. This was done by applying three statistical procedures. The chi square (χ^2) test compared the observed frequency (F_o) of mortality to the expected frequency (F_e) which would have been obtained from a theoretical normal frequency distribution with the same mean ($\bar{x}$) and standard deviation (s). The F_e for any interval can be obtained by converting the limits of the interval into units of s, reading the proportional area under the normal distribution curve from a table of cumulative normal frequency distribution (e.g., 10), and multiplying by the total number of animals dying during the entire experiment. When the exact time of death is not determined but total mortality during a time interval is recorded, the limits of this time interval must be used. For example, the limits of the interval indicated as day 10 are 9.5 to 10.5, and only the midpoint for this period is 10.0. The second and third statistical procedures (10) tested the symmetry of the observed frequency distribution curve and determined whether a symmetrical curve was characterized by a normal or by an abnormal concentration of observations near the center of the range (kurtosis). The results for these tests are presented in Table 2. When the time of death was recorded as the day on which animals died, all three tests indicated a significant deviation from a normal distribution. In some instances, this nonadditivity can be corrected by converting the time of death to its logarithm. When this was done, the deviation from a normal distribution was not significant with respect to the χ^2 and symmetry tests, but the test for kurtosis still revealed significant deviation (Table 2). Therefore, the assumption that survival time was distributed normally among the animal population under the conditions of this experimental model was not valid, and nonparametric methods were used to evaluate the results.

Gehan's generalization of the Wilcoxon test (2) was chosen because it is a nonparametric test which is applicable in a variety of experimental designs involving censored data; i.e., some animals survive the observation period, particularly those receiving chemotherapy (Table 3). The null hypothesis (H_0 = no difference exists between control and treated groups) was tested against the

TABLE 2. *Statistical tests to evaluate the null hypothesis that the frequency distribution of animal deaths (no treatment) approximates the normal distribution curve*

Deaths recorded as	Probability of normal distribution		
	χ^2	Symmetry	Kurtosis
Day of death	<0.01	<0.001	<0.025
Log day of death	>0.10	>0.05	<0.025

TABLE 3. *Survival times for mice infected with Coccidioides immitis, including treated and nontreated groups (controls)*

Groups	Treatment		No. of mice dead on day indicated[a]																				Totals on day 30	
	Dose (mg/kg)	Duration (days)	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Dead	Alive
Controls	0[b]	5			2	5	8	15	9	12	3	4	3	2	1		3		1				68	2
Ampho-tericin B	0.4	5				1	1	4	2	1	4	1	1	1	2								18	2
	1.6	5			1		3	3	1	4	1		1	1			1						16	4
	6.4	5	1	1		2	3	1	4	3	3	2		2	1								23	2
	25.6	5						3	3	4	3	1			3		1	1					19	6
CB-310	4	5						3	4	3	2	2		2			1						17	2
	16	5					5	3	4	1	1	1					1						16	3
	64	5					5	4	6	2	1				1							1	20	5
	256	5						3	1	5	3				4	1	1				1		19	5
	1,024	5							5		1	4											10	0
8308	10	5						1	4	2		1	1										9	1
	10	10							1	5	1	1	1	1									10	0
8309	10	5							2	5	2			1									10	0
	10	10							3	4	1												8	2
8351	10	5						2	4	1	1	1											9	1
	10	10								1	3	1		1	1	1							8	2
4962	10	10					1	3	2	3	1												10	0
8342	40	10								3	2	1			1	1							8	2

[a] No mice died on days 27, 28, 29, and 30.
[b] Injected with either saline or 10% aqueous dimethyl sulfoxide.

alternative (H_1 = there is a difference between control and treated groups) by computing the statistic W, and its variance, $\mathrm{Var}_{H_0}W$. If H_0 is true, then $Z = W/\sqrt{\mathrm{Var}_{H_0}W}$ has a normal distribution with mean 0 and variance 1. With an absolute value of $|Z| \geq 2.58$ for a probability of 0.01, or $|Z| \geq 1.96$ for a probability of 0.05, H_0 would be rejected in favor of H_1, i.e., that animals receiving treatment live longer than control animals. The results of this evaluation are presented in Tables 3 and 4. Mice treated with amphotericin B doses of 25.6 mg/kg survived significantly longer than control animals. The same interpretation applied to the significant effect with CB-310 at 256 mg/kg; the nonsignificant result with this antibiotic at a dose of 1,024 mg/kg was ascribed to known toxicity at this level (*to be published*). The synthetic antifungal chemicals—8308, 8309, 8351, 4962 and 8342—are included to illustrate the use of this model as a method for screening compounds at near maximal tolerated doses (Table 4). Initially, the experimental animals were treated for 5 successive days, but therapy was extended to 10 days with a second group of mice after we learned that this was tolerated well. The use of the experimental model in this manner would serve as a guide indicating that additional investigation might be justified.

DISCUSSION

Current evidence indicates that most humans who are exposed to fungi capable of causing systemic disease develop an acquired immunity early enough to control the slowly evolving mycotic infection (4). Chronic pulmonary or disseminated disease can be attributed to a deficiency in the acquisition of this resistance caused either by an overwhelming initial dose of infecting fungal spores, or by a real immunological incompetence. Chemotherapy, therefore, should be most effective during the early stages of the disease when the exogenous agent might limit the infection until the endogenous resistance can become effective. Unfortunately, presently available antifungal agents are employed only when the infection has progressed to a potentially life-threatening stage. This approach is justified because of the known toxicity of current antifungal agents. A second argument favoring early chemotherapy in symptomatic mycoses is that such cases frequently run a clinical course of several weeks even when complete recovery ultimately ensues. For example, the experience

TABLE 4. *Significance of survival times for treated mice compared with that for control mice (Table 1)*

Groups	Treatment		Probability
	Dose (mg/kg)	Duration (days)	
Amphotericin B	0.4	5	NS[a]
	1.6	5	NS
	6.4	5	NS
	25.6	5	<0.01
CB-310	4	5	NS
	16	5	NS
	64	5	NS
	256	5	<0.01
	1,024	5	NS
8308	10	5	NS
	10	10	<0.05
8309	10	5	NS
	10	10	NS
8351	10	5	NS
	10	10	<0.01
4962	10	10	NS
8342	40	10	<0.01

[a] Not significant.

with coccidioidomycosis at two military bases in Arizona showed that the total time lost because of this disease exceeded that lost due to all other respiratory diseases combined, averaging 34.6 days lost per patient at one base and 33.2 days at the second base (3, 9). Therefore, there is a continuing need for antifungal agents with improved therapeutic efficacy, and there is reason for re-evaluating current practice for chemotherapy of the systemic mycoses.

As noted above, an antifungal agent could be effective even though it were only fungistatic. Furthermore, it would be particularly useful if it were toxicologically safe so that therapy could begin at diagnosis. These considerations suggest that any substance with demonstrable antifungal activity in vitro should be evaluated in an in vivo experimental model, and that this model should be sufficiently sensitive to detect even slight beneficial effects. Since this thesis directs emphasis to the sensitivity of the model, interpretations derived from the results must be qualified by recognizing that the model may be too sensitive and that the evidence indicates only that further experimental animal studies are justified. The particular advantage of a sensitive animal model of the disease lies in reducing the chance that a potentially useful chemotherapeutic agent might be overlooked.

In the present study, the conditions for a suitable experimental animal model of coccidioidomycosis have been satisfied reasonably well. Infected mice exhibited symptomatic disease at the time therapy was instituted, and all were available for treatment. Among the control infected mice (no specific chemotherapy), 97% had died prior to day 30, providing a basis for comparison with treated animals before effective acquired resistance became a significant factor. Appropriate procedures for statistical evaluation were tested for validity. There are, however, certain qualifications with respect to this particular animal model of coccidioidomycosis. Natural infection in this disease occurs after inhalation of spores, and experimental infection via the respiratory route would have been more appropriate. This procedure, however, would have been hazardous for laboratory personnel, as the proper equipment was not available at the time the experiments were performed. Infection via the intraperitoneal route, followed by a delay of 5 days before treatment was started, was a compromise, but it did allow for initiation of extraperitoneal infection before organisms in the peritoneal cavity were exposed directly to antifungal compounds.

The statistical approach employed in these experiments requires comment. Parametric statistics are relatively more efficient than nonparametric methods. The former, however, usually require that the characteristic being measured should be distributed normally in the population studied so that the calculated parameters defining the normal frequency distribution (mean and standard deviation) can be used with confidence in their validity. In animal models of infectious diseases, the frequency distribution curves for survival times commonly deviate from the normal distribution curve, either by asymmetry (skewness) or by kurtosis (symmetrical curve but with too many or too few observations concentrated about the center of the range). If one wishes to use the more efficient parametric statistical methods, then one is obliged to determine whether such a deviation exists and, if so, whether the deviation from normal is great enough to invalidate analyses by usual normal statistics. The alternative would be to use the nonparametric methods which make no assumptions regarding distribution. Gehan's (2) generalization of the Wilcoxon test is particularly useful for studies comparing two treatments when observations are made in time to failure (death of the animal in this study) or censoring (survival

for 30 days in this case). The method is applicable to a variety of experimental situations: with subjects started on trial simultaneously and for a fixed time, in the same situation but with random replacement of subjects that fail during the trial period, and with subjects entering the study serially after time zero. The last application should be particularly useful for clinical studies where patient relapse could be recorded as failure and patient remission as censoring from the study.

Several examples of the application of this experimental model have been presented (Table 4). With amphotericin B as an antifungal antibiotic of known efficacy a significant increase in survival time was apparent with the 25.6 mg/kg dose. This is comparable to results obtained by other investigators for experimental candidiasis, coccidioidomycosis, cryptococcosis, and histoplasmosis, even though the experimental designs differed (7, 11). With a still impure preparation of a new antibiotic, CB-310, significant activity was found, but at drug concentrations uncomfortably close to toxic levels. Perhaps further refinement of this product is justified, and subsequent testing might reveal a wider margin of chemotherapeutic safety. In the third example with the synthetic organoselenium compounds, the experimental model was used as a screening procedure to detect in vivo activity among chemicals with in vitro antifungal activity. The molecular structure of these would be modified in a systematic attempt to increase antifungal potency and reduce toxicity and to determine the molecular basis for these properties.

This animal model, therefore, proved to be sensitive enough to detect even slight fungistatic activity in an in vivo environment, which was the primary objective of the current study. It should be noted that there were significant increases in survival times among treated mice, even though the percentage of animals surviving for 30 days in the several groups did not differ greatly (Tables 3 and 4). Prolonging the period of therapy up to the beginning of an efficient acquired resistance should result in increased numbers of survivors, as predicted by the original hypothesis. This is the subject of continuing investigation.

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