In Vitro and In Vivo Activity of Hamycin Against
Blastomyces dermatitidis

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Clinical responses of patients with blastomycosis to treatment with hamycin have been variable. An explanation for this was sought in a series of studies in which in vitro and in vivo susceptibilities to hamycin of five strains of Blastomyces dermatitidis were compared. Minimal inhibitory concentrations of hamycin for the five strains indicated uniformly high levels of in vitro susceptibility (0.008 to 0.016 μg/ml). In vivo activity was measured in infected mice treated intraperitoneally for a period of 28 days with doses of the drug ranging from 0.001 to 0.030 mg per mouse. Significant differences in response to treatment among the five strains were noted (P < 0.001), and protective doses were found to vary from 0.001 to > 0.030 mg per mouse per day. Further observations of infected mice after treatment revealed marked rates of relapsing infection, and several strains caused death. Persistent inapparent infections were also detected on culture of selected organs. Toxicity due to hamycin alone was not observed. These results suggest that variations in clinical responses to hamycin therapy in treatment of blastomycosis reflect differences in pathogenesis and host response in vivo to the infecting organism rather than differences in susceptibility of B. dermatitidis to hamycin.

Hamycin, a polyene antibiotic produced by Streptomyces pimprina (7), is closely related chemically to amphotericin B (3) but differs from the latter drug in that it is well tolerated and in that prolonged antifungal concentrations in blood are obtained after oral administration (5). It has been shown to be active in vivo in experimental infections with several species of pathogenic fungi, including Cryptococcus neoformans (2, 6, 10), Histoplasma capsulatum (2, 10), Blastomyces dermatitidis (10, 11), and Coccioides immitis (10).

The ability to produce effective antifungal concentrations in serum by oral administration suggests that hamycin might be potentially superior to amphotericin B by this route in the treatment of systemic mycotic infections in man. However, results of clinical trials with this drug have been variable. In one such study, seven patients with blastomycosis were treated with the drug in the form of a pressed tablet. Total drug intake was from 99 to 159 g, and mean concentrations of hamycin in serum from 0.02 to 0.04 μg/ml were obtained (8). Apparent cures were reported for two patients, and a third patient showed clinical improvement but continued positive cultures were obtained. The remaining four patients, all described as having more severe infections, did not respond to treatment.

More recently, a micronized preparation of hamycin was evaluated in five patients with blastomycosis and two patients with histoplasmosis (9). In this study, the patients were treated, in a crossover fashion, with both the tableted and the newer micronized preparations. Apparent cures were obtained in four of the patients with blastomycosis and in one of the patients with histoplasmosis. The remaining patients improved clinically but remained culturally positive. Concentrations of hamycin in serum were found to be higher during treatment with the micronized preparation than with an equivalent dose of the tableted preparation.

Two variables seem to be involved in the above clinical studies with hamycin. In the first study, severity of infection appeared to be the main factor in determining whether or not hamycin therapy was successful. In the second study, the one patient with blastomycosis who failed to

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1 Portions of this paper were presented at the 67th Annual Meeting, American Society for Microbiology, New York, N.Y., 30 April–4 May 1967 (Bacteriol. Proc., p. 72, 1967).
respond to treatment with hamycin was found to have lower concentrations of the drug in serum than those patients in whom complete cures were obtained. This suggests that inadequate concentrations in serum may have been involved in the various therapeutic failures. However, there remains a third variable which must be considered, and that is the possibility of inherent differences in susceptibility of different strains of *B. dermatitidis* to this antifungal agent. This study was therefore undertaken to determine and compare the following for several strains of *B. dermatitidis*: (i) in vitro susceptibilities to hamycin, (ii) in vivo responses to hamycin therapy, and (iii) the correlation between in vitro susceptibilities, in vivo responses, and clinical responses obtained with hamycin in those patients from whom the strains under study were originally isolated.

**MATERIALS AND METHODS**

Five strains of *B. dermatitidis* were studied. These included four clinical isolates obtained prior to treatment with hamycin and one reference strain. The clinical isolates are designated by patient's initials, i.e., G. B., R. L., E. W., and W. W. Pertinent clinical data concerning the patients from whom these strains were recovered are summarized in Table 1 (8). The reference strain, designated NIH, was obtained from the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

**Cultures.** Yeast-phase cultures of the five strains of *B. dermatitidis* were obtained after a series of rapid serial subcultures at 37° C on Brain Heart Infusion (BHI) Agar (Difco) with 5% sheep blood and were maintained in a biphasic medium consisting of BHI Agar with sheep blood and an overlay of BHI broth (Difco). For several experiments, strains G. B., R. L., and NIH were propagated in the yeast phase in fluid shake cultures at 37° C in a synthetic medium described by Gilardi and Laffér (4). This medium had the following composition (in grams per liter): glucose, 10.00; (NH₄)₂SO₄, 5.00; KH₂PO₄, 1.00; MgSO₄·7H₂O, 0.50; CaCl₂·2H₂O, 0.10; and NaCl, 0.10.

**In vitro susceptibility studies.** Minimal inhibitory concentrations (MIC) of hamycin for the five strains of *B. dermatitidis* were determined by means of a tube dilution procedure employing descending concentrations of hamycin in BHI Agar supplemented with 5% sheep blood. Hamycin (supplied as a standard reference powder by Sherman Laboratories, Detroit, Mich.) was prepared initially as a 100,000 μg/ml solution in dimethyl sulfoxide (DMSO), diluted to 10,000 μg/ml in 60% ethyl alcohol, and further diluted to a concentration of 1,000 μg/ml in sterile saline. This solution was then serially diluted in sterile saline, and the resulting dilutions were mixed with nine parts of sterile BHI Agar, dispensed in sterile screw-cap test tubes, and slanted. Final concentrations of the drug were from 0.001 to 100 μg/ml in final volumes of 5.0 ml.

Yeast-phase cultures of the five strains of *B. dermatitidis* were propagated on the biphasic medium described above. The mature cultures were harvested and concentrated in sterile saline after filtration through sterile cotton and centrifugation. The resulting suspensions were then diluted in sterile BHI broth and inoculated in 0.1-ml volumes onto the prepared tubes of hamycin in BHI Agar. Four tubes were inoculated for each strain at each level of drug concentration; four drug-free control tubes were also included for each strain.

The inoculated tubes were incubated at 37° C for 72 hr. Growth responses were determined on a graded basis of 0 to 4+, with 1+ presenting a minimal response of fewer than 10 distinct colonies and 4+ being equivalent to growth obtained on the drug-free BHI Agar control tubes. The MIC was defined as the lowest concentration of hamycin which completely inhibited the growth of *B. dermatitidis* in all four tubes.

**In vivo studies.** Susceptibilities of the five strains of *B. dermatitidis* to hamycin were measured in vivo by determining the minimal effective concentrations of hamycin in vivo.
in experimentally infected mice. Yeast-phase cultures of strains W. B. and E. W. were harvested from BHI Agar with 5% sheep blood and were concentrated in sterile saline. Strains G. B., R. L., and NIH were grown in the synthetic medium of Gilardi prior to concentration in normal saline.

The concentrated suspensions were diluted to contain 80,000 to 100,000 cells/ml as measured by direct microscopic counts in a Petroff-Hausser chamber. Total viable counts were not determined. For each of the five strains of B. dermatitidis, 150 ICR-originated mice (Maryland Breeders for Research, Burtonsville, Md.) weighing 20 to 22 g were inoculated via the lateral tail vein with 0.5 ml of the adjusted suspension. Prior to inoculation, it had been determined that such inocula generally produced lethal infections within 14 days with this strain of mice. Treatment with hamycin was started 72 hr after the initial infectious challenge.

The hamycin was prepared as a concentrated solution in 10% DMSO in sterile distilled water and was frozen at −35 C. Samples were thawed and diluted as required. Diluted solutions were used for a maximum of 3 days and stored at 4 C. Ten dose levels ranging from 0.001 to 0.030 mg/mouse (0.05 to 1.5 mg/kg) were tested; 10 mice were included at each dose level for each of the five strains of B. dermatitidis. The drug was administered intraperitoneally in 0.5-ml volumes 5 days a week for 4 weeks. Controls included, in addition to the appropriate normal and infected animals with and without treatment, normal animals inoculated according to the same schedule with a 5% solution of DMSO in sterile distilled water. This inoculum was equal to the highest concentration of DMSO received by any of the treated animals.

Those animals which survived the initial 28 days of treatment were further observed for an additional 34 days to permit detection of relapsing infections. At the end of the second period of observation, all remaining mice were killed and examined for the presence of signs of active infections. Samples of liver, spleen, and lung were obtained for microscopic and cultural examination. Noninfected, treated mice were also killed at this time, and portions of spleen, liver, lung, and kidney tissues were removed and placed in 10% buffered Formalin for subsequent histopathological examinations. Specimens intended for cultural studies were frozen at −35 C; subsequently, these specimens were thawed and ground in sterile saline, and the resulting suspensions were plated on Mycosel agar (BBL). The inoculated plates were incubated for up to 4 weeks at 30 C.

RESULTS

In vitro sensitivity studies. The five strains of B. dermatitidis were found to be extremely susceptible to hamycin (Table 2). Furthermore, the range of susceptibility was narrow: 0.008 to 0.016 μg/ml, with a mean value of 0.012 μg/ml and a standard deviation of 0.004 μg/ml. Differences between MIC values for the different strains were not significant. All readings were taken after 72 hr of incubation, as further incubation at 37 C was found to result in measurable decay of the drug. Growth of B. dermatitidis occurred after 96 to 144 hr of incubation in previously negative tubes containing 0.016 μg of hamycin per ml or more.

In vivo studies. Ten closely spaced concentrations of hamycin were employed in treatment of experimental infections in mice with the five strains of B. dermatitidis. However, variations in therapeutic responses are best seen if data for only 4 of the 10 dose levels and for the untreated infected control animals are examined (Fig. 1–5).

In untreated mice, infections with strains G. B., W. B., R. L., and E. W. were uniformly severe (Fig. 1). Death occurred in 50% of the untreated mice within 5 to 10 days, and the infection was fatal in all of these mice within 3 weeks. Differences in fatality rates were not significant among these four strains, nor were there significant differences in fatality rates produced by strains propagated on BHI Agar as compared

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimal inhibitory concn</th>
</tr>
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<tbody>
<tr>
<td>G. B.</td>
<td>0.008</td>
</tr>
<tr>
<td>W. B.</td>
<td>0.012</td>
</tr>
<tr>
<td>R. L.</td>
<td>0.016</td>
</tr>
<tr>
<td>E. W.</td>
<td>0.008</td>
</tr>
<tr>
<td>NIH</td>
<td>0.016</td>
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</tbody>
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* Y = 0.012 μg/ml; standard deviation = 0.004.

![Fig. 1. Mortality in mice experimentally infected with five strains of B. dermatitidis with no treatment.](http://jb.asm.org/)

![Table 2. Minimal inhibitory concentrations of hamycin for five strains of Blastomyces dermatitidis](http://jb.asm.org/)
with those propagated in the liquid synthetic medium. With strain NIH, the 50 and 90% fatality levels were not attained until the 4th and 6th weeks, respectively.

In treated mice, evidence of therapeutic activity of hamycin, as indicated by extended survival time, was first observed in mice infected with strains R. L., E. W., and NIH which were treated with the dosage of 0.005 mg per mouse per day (Fig. 2). At the end of the 28 days of treatment, survival rates for mice infected with these three strains were, respectively, 33, 100, and 100%.

At doses of 0.010 mg/day, mice infected with strain E. W. were fully protected during the 4 weeks of treatment, and deaths were not observed until the 3rd week post-treatment (Fig. 3). With strains G. B. and R. L., fatalities were first observed in the 2nd and 6th weeks, respectively. Mice infected with strain NIH were completely protected; no deaths occurred during the 9 weeks of treatment and observation. With strain W. B., the fatality rate was essentially unchanged from that of the untreated infected controls.

Maximal therapeutic results were obtained with daily doses of 0.020 mg/day (Fig. 4). In mice infected with strains E. W. and NIH, no deaths were observed during either the treatment or observation periods. With strains G. B. and R. L., fatality rates were reduced to 10 and 11%, respectively, at the end of treatment. However, there were deaths in the former mice following termination of treatment. The overall fatality rate was unchanged in mice infected with strain W. B.

Results were not as good in mice treated with the highest dose of 0.030 mg/day (Fig. 5). Particularly in mice infected with strains NIH and G. B., the fatality rates were greater than those

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Fig. 2. Mortality in mice experimentally infected with five strains of B. dermatitidis and treated with 0.005 mg of hamycin per mouse per day for 28 days.

Fig. 3. Mortality in mice experimentally infected with five strains of B. dermatitidis and treated with 0.010 mg of hamycin per mouse per day for 28 days.

Fig. 4. Mortality in mice experimentally infected with five strains of B. dermatitidis and treated with 0.020 mg of hamycin per mouse per day for 28 days.
obtained with 0.020 mg per mouse per day after the second week of treatment (Fig. 6).

Gross and cultural examinations of tissues following sacrifice of all mice surviving after the 9th week indicated that in the majority of animals treatment with hamycin failed to eliminate the initial infections completely. Gross pulmonary lesions were observed in 38% of the 171 surviving mice, and B. dermatitidis was recovered from 61% of these mice (Table 3). No correlation was seen between recovery of viable cells of B. dermatitidis from tissues and dosage of hamycin in mice infected with strains G. B. and R. L. Only with strains E. W. and NIH at doses of 0.010 mg/day or more were a majority of the mice negative on either examination or culture.

Toxicity. Only two deaths were observed in noninfected mice treated with hamycin. One occurred during the 1st week of treatment in the group receiving 0.030 mg per mouse per day, and the other occurred during the 6th week in the group receiving 0.001 mg per mouse per day. The absence of deaths in mice receiving amounts up to 0.025 mg/day suggests that the latter death most probably resulted from causes other than toxicity. No histological evidence of toxicity was seen in spleen, liver, lung, or kidneys of the remaining noninfected animals treated with the higher doses.

**DISCUSSION**

The purpose of these studies was to demonstrate any associations which might exist between in vitro and in vivo susceptibilities of several strains of B. dermatitidis to hamycin and clinical responses obtained with this drug in the four patients from whom the strains were originally isolated. Such associations do not appear to exist. Analysis of the results of the in vitro susceptibility determinations revealed a uniform degree of susceptibility to hamycin without significant variation for the four clinical isolates and the reference strain of B. dermatitidis. In the in vivo studies, strain W. B., isolated from the only one of the four patients who was cured clinically, was found to be the most resistant to hamycin whereas strain E. W., isolated from a patient whose disease did not respond to hamycin, was the most susceptible.
In contrast, significant differences can be demonstrated among the strains in their in vivo responses to hamycin in experimentally infected mice. These differences cannot be attributed to detectable variations in severity or lethality of the experimental infections. Comparison of fatality rates in nontreated, infected mice reveals no significant differences in lethality among the infections produced by the five strains \((P = 0.80)\). Further, the differences in therapeutic responses in infected mice, as in clinical cases, do not appear to be related to variations in the in vitro susceptibility to hamycin. An examination of these differences in light of their possible clinical and therapeutic significance is warranted.

Comparison of the dosages of hamycin required to protect 50% of mice \(\left(\text{PD}_{50}\right)\) infected with the four clinical isolates of \(B. \text{dermatitidis}\) indicates the extent of the variation in the in vivo therapeutic responses. The \(\text{PD}_{50}\) values ranged from 0.005 mg per mouse per day for mice infected with strain E. W. to more than 0.030 mg per mouse per day for mice infected with strain W. B. No associations can be demonstrated when these results are compared by rank order correlation with in vitro susceptibility values (Table 4). In addition, comparison of fatality rates obtained when the various dosages were administered to mice infected with the different strains reveals significant variations in the distribution of deaths throughout the entire dose range \((P < 0.01)\).

The significance of the differences in the distribution of deaths in infected mice treated with hamycin in amounts either less than or greater than the mean \(\text{PD}_{50}\) value for 28 days of treatment \((0.011 \text{ mg per mouse per day})\) was tested. Comparison of death rates for strains E. W., R. L., G. B., and W. B. within composite groups consisting of all mice for each strain treated with low doses of hamycin, 0.0 to 0.010 mg/day, or with high doses, 0.0125 to 0.030 mg/day, showed the differences within the composite rankings to be significant \((x^2 \text{ analysis, } P < 0.001)\). This comparison also revealed possible similarities in either pathogenicity or in vivo responses to hamycin between several of the strains of \(B. \text{dermatitidis}\). The distributions of deaths in the composite treatment groups for strains W. B. and G. B. differ significantly from those of R. L. and E. W. but not from each other.

Analysis of rates of relapsing infections with deaths following treatment and for the persistence of gross lesions or retention of viable cells of \(B. \text{dermatitidis}\) at the end of the experiment also reveals significant differences between strains. The differences in relapse rates were tested, according to the Wilcoxon pairs test, by comparing deaths observed during treatment with those following treatment. The results revealed that the death rate in mice infected with strain R. L., but not the other three strains, increased significantly \((P < 0.01)\) following termination of treatment. Rates of persistence of gross lesions and for retention of viable cells of \(B. \text{dermatitidis}\) were subjected to partition analysis by use of the Brandt-Snedecor formula (1). This analysis reveals significant differences \((P < 0.001)\) among the strains in both respects. Further analysis demonstrates that the differences were due for the most part to strains G. B. and R. L. Partition analysis showed the variations in the rates of persistence of gross lesions among strains R. L., E. W., and NIH but exclusive of strain G. B. to be nonsignificant \((P < 0.5)\). Moreover, similar analysis of the variations in rates of retention of viable fungi revealed significant differences when strains R. L. and G. B. were compared, as a group, with strains E. W. and NIH \((P < 0.001)\). When the differences in rates for retention of viable fungi in tissues of those mice with persistent lesions were tested, only strain R. L. was found to be significantly different \((P < 0.001)\) for all strains including R. L.; \(P > 0.20\) for all strains excluding R. L.).

As noted above, this was the only strain for which a significant rate of relapsing infections was demonstrated. It should be noted also that therapeutic failures followed treatment with both hamycin and amphotericin B in patient R. L.

The levels of chemotherapeutic activity of hamycin against \(B. \text{dermatitidis}\) reported here are similar to those reported by Williams and Emmons (11). With intraperitoneal doses of 0.0196 and 0.0274 mg per mouse per day, these authors reported both protection and sterilization of tissues. Williams, Bennett, and Emmons (10) demonstrated both protection and apparent

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**Table 4. Comparison of 50% protective doses and minimal inhibitory concentrations of hamycin for five strains of Blastomyces dermatitidis**

<table>
<thead>
<tr>
<th>Strain</th>
<th>50% protective dose</th>
<th>Minimal inhibitory concn</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1-28 days</td>
<td>1-62 days</td>
</tr>
<tr>
<td>W. B.</td>
<td>&gt;0.030</td>
<td>&gt;0.030</td>
</tr>
<tr>
<td>G. B.</td>
<td>0.0125</td>
<td>0.029</td>
</tr>
<tr>
<td>R. L.</td>
<td>0.0075</td>
<td>0.0125&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. W.</td>
<td>0.005</td>
<td>0.010</td>
</tr>
<tr>
<td>NIH</td>
<td>0</td>
<td>0.005</td>
</tr>
<tr>
<td>Mean</td>
<td>0.011</td>
<td>0.016</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by graphic analysis.
<sup>b</sup> Higher mortality at 0.030 mg/day.
<sup>c</sup> Mortality of 67% at 0.015 mg/day.
sterilization of tissues in mice severely infected with *B. dermatitidis* and treated intraperitoneally with 0.0320 to 0.0553 mg per mouse per day.

Some evidence of chronic drug toxicity was observed. In mice infected with strains G. B. and R. L., mortality rates increased when the daily dose of hamycin exceeded 0.020 mg. In contrast, except for a single death occurring during the 1st week at the 0.030 mg/day dosage, no such toxicity was observed in the uninfected animals. The possible significance of these increases was tested by comparing times of death in mice treated with 0.020 mg/day with those in mice treated with 0.030 mg/day. The difference was significant for strain G. B. (Kolomogrov Smirnov test, *P* < 0.01) but not for strain R. L. Differences in days of death between infected mice receiving no treatment and infected mice treated with either 0.020 or 0.030 mg/day were also tested. While the differences between mortality rates in nontreated mice and in mice treated with 0.020 mg/day were highly significant for all five strains (Kruskal Wallis test, *P* < 0.001), the levels of significance were reduced at the 0.030 mg/day dose for strains W. B. (*P* < 0.01), G. B. (*P* < 0.05), and NIH (not significant). This phenomenon of increased deaths with higher dosages of hamycin in only the infected animals has also been described by Williams and Emmons (11). The absence of toxicity in noninfected animals treated with comparable doses suggests a mechanism other than simple drug toxicity. Such a mechanism might include release of toxic fungal cell components following exposure to hamycin in situ. This suggestion is supported by the absence of histological evidence of toxicity in noninfected treated animals.

The studies reported here did not demonstrate any association between clinical responses to hamycin in cases of blastomycosis and the results of in vivo and in vitro susceptibility studies with *B. dermatitidis*. However, they did reveal significant differences in the in vivo responses of several strains of *B. dermatitidis* to hamycin. Although these differences may be due in part to undetected differences in virulence among the strains, it is concluded that they represent valid differences in the complex interactions between fungal pathogen, host, and antifungal agent.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**