In Vitro Susceptibility of Pathogenic Naegleria and Acanthamoeba Species to a Variety of Therapeutic Agents

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Six pathogenic strains of Naegleria fowleri, two of Acanthamoeba castellanii, and three of Acanthamoeba polyphaga were tested in vitro for susceptibility to a variety of potentially useful therapeutic agents. Minimal motility inhibitory concentrations and minimal inhibitory concentrations were determined by a technique of subculturing pure clones of amoebae in plastic tissue culture chamber slides containing liquid axenic media and serially diluted drug, incubating at 30°C for Acanthamoeba and at 37°C for Naegleria, and observing on an inverted microscope at 6 h for inhibition of motility and at 24 and 48 h for inhibition of growth. Drug concentrations were selected on the basis of fluid levels achievable in humans. Amphotericin B, clotrimazole, and miconazole were the most effective drugs against Naegleria, whereas polymyxin B sulfate and pentamidine isethionate were somewhat effective against pathogenic Acanthamoeba. Our results suggest that amphotericin B is the most effective agent against Naegleria, but few agents are effective against Acanthamoeba.

Not until the 1960s was the pathogenicity of free-living amoebae fully appreciated, that is, when the disease primary amoebic meningoencephalitis (PAM), an acute, rapidly fatal infection occurring in otherwise healthy humans, was recognized (3, 15). In this disease victims are children or young adults who have been swimming in or exposed to fresh or brackish waters; the responsible pathogen is a free-living amoeboid-flagellate belonging to the genus Naegleria, specifically, Naegleria fowleri (14). Amoebae enter the nose and during the subsequent 5 to 6 days migrate through submucosal structures and nerves, ultimately invading the subarachnoid space and brain substance beneath the cribriform plate. Within 72 h rapid deterioration of the patient ensues, resulting in coma and death. An almost identical disease may be produced experimentally in mice by intranasal instillation of these same amoebae (20).

In addition to Naegleria, within the last few years, amoebae of the genera Acanthamoeba and Hartmannella also are being increasingly incriminated as a cause of meningoencephalitis (14, 16, 23) and, in addition, as a cause of nonfatal infections, such as ophthalmitis (21; D. B. Jones, G. S. Visvesvara, and N. M. Robinson, Oxford Ophthalmological Congress, Oxford, England, 1975) and otitis (17). Experimental infections in mice with Acanthamoeba-Hartmannella spp. can also be produced (8, 10, 19); however, in contrast to Naegleria, when Acanthamoeba sp. are instilled intranasally, pneumonia is often the principal disease, and an incubation period longer than 7 days is usually present (19). Spread to the central nervous system occurs principally via the blood stream and generally has resulted in chronic (occasionally granulomatous) meningoencephalitis and minimal, if any, meningitis.

Because of the serious nature of both Naegleria and Acanthamoeba-Hartmannella infections in humans and because of an almost complete lack of therapeutic agents available against these pathogens, a variety of drugs reported or demonstrated to have antiprotozoan activity was examined.

MATERIALS AND METHODS

Pure clones of six strains of pathogenic Naegleria and five strains of Acanthamoeba were studied (Table 1). All strains of Naegleria were identified as N. fowleri and were from fatal cases of human PAM, and all were capable of producing 70 to 100% mortality in mice when approximately 10⁴ organisms were instilled intranasally. Three isolates (L.L., T.Y., and W.M.) were obtained from patients seen in 1967 and 1969 at the Medical College of Virginia Hospitals (13); two (F-66 and F-69) were from patients seen in Australia in 1966 and 1969 (5), courtesy of Rodney F. Carter; and one (G.J.) was from a 23-month-old child who died in Florida in 1973, courtesy of Eugene Meagor and Shih L. Chang. Of the five strains of Acanthamoeba, two were Acantha-
moeba castellanii. (The Gresham strain was from a human ocular infection [21], courtesy of J. Nagin- 
ton and Frederick Page, Cambridge, England; the 
CH-6 strain was from a freshwater lake in Kentucky, 
courtesy of Shih L. Chang; it was capable of produc-
ing up to 60% mortality in mice inoculated intra-
nasally with 10^4 organisms [9]). Three strains were A. 
polyphaga. (The Garcia strain was from a human 
ocular infection [Jones et al., Oxford Ophthalmological 
Congress, 1975], courtesy of Daniel B. Jones; the 
CH-5 strain was a contaminant of tissue culture, 
courtesy of Dr. Chang; and the P-6 strain was found in 
as series of freshwater isolates collected in the 
United States by Frederick Page.)

Table 1. Strains of free-living amoebae studied

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Year of isolation</th>
<th>Investigators</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. fowleri, F-66</td>
<td>Human PAM, Australia</td>
<td>1966</td>
<td>Fowler and Carter</td>
</tr>
<tr>
<td>N. fowleri, F-69</td>
<td>Human PAM, Australia</td>
<td>1969</td>
<td>Fowler and Carter</td>
</tr>
<tr>
<td>N. fowleri, W.M.</td>
<td>Human PAM, Virginia</td>
<td>1969</td>
<td>Duma and Nelson</td>
</tr>
<tr>
<td>N. fowleri, L.L.</td>
<td>Human PAM, Virginia</td>
<td>1968</td>
<td>Duma and Nelson</td>
</tr>
<tr>
<td>N. fowleri, T.Y.</td>
<td>Human PAM, Florida</td>
<td>1973</td>
<td>Meager and Chang</td>
</tr>
<tr>
<td>A. castellanii, Gresham</td>
<td>Ocular infection, England</td>
<td>1974</td>
<td>Nagington</td>
</tr>
<tr>
<td>A. castellanii, CH-6</td>
<td>Fresh water, Kentucky</td>
<td>1969</td>
<td>Chang</td>
</tr>
<tr>
<td>A. polyphaga, Garcia</td>
<td>Ocular infection, Texas</td>
<td>1974</td>
<td>Jones</td>
</tr>
<tr>
<td>A. polyphaga, CH-5</td>
<td>Tissue culture, Puerto Rico</td>
<td>1972</td>
<td>Wang and Chang</td>
</tr>
<tr>
<td>A. polyphaga, P-6</td>
<td>Fresh water, United States</td>
<td>?</td>
<td>Page</td>
</tr>
</tbody>
</table>

The therapeutic agents tested were as follows: 

- amphotericin B (E.R. Squibb & Sons, Inc., Prince-
ton, N.J.) dissolved in dimethyl sulfoxide (range, 
maximum of 3.4% for 50 μg/ml to 0.006% for 0.098 μg/ml); 
- clotrimazole or Bay 5097 (Delbey Pharmaceuti-
cals, Inc., Bloomfield, N.J.) dissolved in di-
methyl sulfoxide (range, maximum 2% for 100 μg/ 
ml to 0.004% for 0.195 μg/ml); 
- metronidazole (Searle & Co., San Juan, Puerto Rico) 
dissolved in N,N-
dimethyl formamide (highest concentration, 0.120%); 
- sulfamethoxazole (Hoffmann-La Roche, Inc., Nut-
ley, N.J.) dissolved in 0.1 N sodium hydroxide 
(highest concentration, 0.01%); 
- trimethoprim (Bor-
roughs-Wellcome, Research Triangle Park, N.C.) 
dissolved in 0.1 N hydrochloric acid (highest 
concentration, 0.01%); 
- a 20:1 mixture of sulfamethox- 
azole and trimethoprim; 
- miconazole (Janssen R & D, Inc., New Brunswick, N.J.) plus micona-
zel placebo (0.115 ml of polyethoxylated castor oil, 0.5 
mg of sodium bisulfite, and 1.62 mg of methylpara-
ben per 1 ml); 
- polymyxin B sulfate (Pfizer Labora-
tories, New York, N.Y.); 
- pentamidine isethionate (Center for Disease Control, Atlanta, Ga.); 
- para-
omycin sulfate (Parke, Davis & Co., Detroit, 
Mich.); 
- G-418 (Schering Corp., Bloomfield, N.J.); 
- 5-fluorocytosine (Hoffmann-La Roche, Inc., Nutley, N.J.); 
- and clindamycin hydrochloride, as well as its 
derivative U34728E (The Upjohn Co., Kalamazoo, 
Mich.), all dissolved in sterile distilled water. The 
top concentration of metronidazole was 250 μg/ml; 
for amphotericin B, 50 μg/ml; and for all other 
agents, 100 μg/ml. All tests were conducted in 
triplicate.

To test drug susceptibility, a liquid, bacteria-free 
alcohol (axenic) in vitro test system was developed and util-
ized (Fig. 1). Agents to be studied were solubilized in 
stock solutions from which maximum concentra-
tions and subsequent twofold dilutions were made in 
modified Nelson's axenic liquid nutrient medium 
[22]. The pH of this medium with and without drugs 
was between 7.2 and 7.4. Using an automatic non-
electric pipette (BBL, 60422), 0.4 ml of each drug 
concentration (obtained by making twofold serial 
dilutions of the drug in axenic media) was placed in 
chambers of an eight-chambered Lab-Tek tissue cul-
ture chamber/slide (Miles Laboratories, no. 4808). 
Again, using the automatic non-electric pipette, 0.1-
ml suspensions containing 10^6 trophozoites from 48-
to 72-h 37°C liquid axenic cultures of Naegleria or 
from 30°C liquid axenic cultures of Acanthamoeba 
were added to each chamber. (The decision to use 10^6 
amoebae/0.1 ml was made to provide microscopic 
fields of approximately 25 to 40 organisms when a 
25× ocular and 10× objective of an inverted, Ameri-
Can Optical, ordinary light microscope were used.) 

To one chamber, as appropriate controls, amoebae 
were added to axenic media, containing, if neces-
sary, a concentration of solvent equivalent to that 
used in the maximum drug concentration studied.

After incubation at either 37 or 30°C (depending 
on the species of amoeba being studied) for 6 h, 
minimal motility inhibition concentrations (MMIC) 
were determined by microscopic examination and 
comparison with controls. Immobilization of tropho-
zoites was generally accompanied by certain mor-
phological changes, as described previously [12]: 
diminution in size, rounding up, watery ectoplasm, 
increase in granulation, and/or complete disintegra-
tion of amoebae. The MMIC was defined as the 
lowest concentration of drug at which no active 
pseudopodial activity was seen as compared with 
controls. After reincubation of the chamber slide for 
24 h and again for a total of 48 h, the minimal 
inhibitory concentration (MIC) of growth was deter-
mined. The MIC was defined as the lowest 
concentration of drug at which growth was 50% less than 
that of the control, as determined visually on the 
inverted microscope (Fig. 2).

All susceptibility tests were run in triplicate 
(with one exception), and geometric means were 
calculated for the MMIC and 24- and 48-h MICs 
against each strain for every drug.
FIG. 1. Schematic representation of in vitro technique utilized for antiamoebic susceptibility testing (see Materials and Methods for explanation).

FIG. 2. Microscopic examination of four chambers containing amphotericin B and a liquid axenic culture of Naegleria. The surface of the control chamber (upper left) is covered with viable, motile amoebae, whereas organisms in a chamber containing 0.39 μg/ml are beginning to round up and lose their motility (lower right). At 0.78 μg/ml (lower left) few remnants of amoebae are visible, and at 1.56 μg/ml (upper right) viable amoebae are absent.
RESULTS

The results of this investigation confirmed earlier observations utilizing monaxenic cultures (containing Enterobacter as the sole source of nutrient for amoebae) that amphotericin B is still the most effective drug currently available against Naegleria. Mean MMICs (Table 2) against the six strains ranged from 0.98 to 1.97 \( \mu g/ml \), with a mean MMIC for the group of 1.39 \( \mu g/ml \), whereas mean MICs at 24 h ranged from 0.39 to 1.24 \( \mu g/ml \), with a mean MIC for the group of 0.62 \( \mu g/ml \). Mean MICs of amphotericin B at 48 h against these same six strains ranged from \( \leq 0.15 \) to 0.39 \( \mu g/ml \), with a mean for the total of \( \leq 0.26 \mu g/ml \). Differences in experimental results against any one strain never exceeded two dilutions of drug and were within one dilution in every instance but one. It was noted for amphotericin B that the 6-h MMIC values predicted 24- and 48-h growth inhibitory activity (MIC) in every instance.

Clotrimazole and miconazole were also effective against Naegleria, but their effect was not as rapid as was that of amphotericin B. For example, mean MMICs determined 6 h after exposure to clotrimazole were 50 \( \mu g/ml \) against all six strains. In addition, concentrations required to inhibit growth varied considerably from strain to strain, as mean 24-h MICs of clotrimazole against these same strains ranged from \( \leq 0.39 \mu g/ml \) to as high as 39.69 \( \mu g/ml \) (Table 2). Similar results were observed for miconazole, as mean MMICs were always above 39.69 \( \mu g/ml \), whereas mean 24-h MICs ranged from 0.78 to 25 \( \mu g/ml \). Mean 48-h MICs for both clotrimazole and miconazole fell below those values observed at 24 h, ranging from \( <0.39 \) to \( \leq 1.57 \mu g/ml \) and 0.98 to 1.97 \( \mu g/ml \), respectively. In contrast to amphotericin B, 6-h MMICs of clotrimazole or miconazole did not often help in predicting which Naegleria strain would be inhibited. Again, with the test system used, triplicate determinations of 24- or 48-h MICs were in almost every instance within two dilutions (or not statistically significant).

No other therapeutic agents tested showed any inhibition of either motility or growth of Naegleria in the concentrations tested.

With strains of Acanthamoeba, of all agents tested, only polymyxin B and pentamidine isethionate appeared to be effective (Table 3), but only slightly so, with 6-h MMICs ranging from 50 to \( \geq 100 \mu g/ml \) (mean, 50.15) and from 35.36 to \( \geq 70.71 \mu g/ml \) (mean, 54.27), respectively. MICs of polymyxin B at 24 h were 50 to \( \geq 100 \mu g/ml \) (mean, 72.36), and at 48 h they were 79.37 to \( \geq 100 \mu g/ml \) (mean, 87.06). MICs for pentamidine at 24 h ranged from 35.36 to \( \geq 100 \mu g/ml \) (mean, 45.85), and at 48 h they were from 12.5 to 50 \( \mu g/ml \) (mean, 22.93).

**DISCUSSION**

Based on this study and on prior in vitro data (4, 9, 12, 24), amphotericin B still appears to be the most reliable and effective agent against pathogenic strains of Naegleria. In this study, mean MMICs ranged from 0.98 to 1.97 \( \mu g/ml \) for the six pathogenic strains tested, and 24- and 48-h MICs ranged from 0.39 to 1.24 and from \( \leq 0.15 \) to 0.39 \( \mu g/ml \), respectively. Earlier observations in this laboratory, utilizing crude monaxenic cultures of five strains of pathogenic Naegleria grown with Enterobacter aerogenes as the sole source of nutrient, indicated that amphotericin B inhibited motility at 6 h at a concentration of \( \leq 0.625 \mu g/ml \) and was amebicidal at concentrations of \( \leq 0.078 \mu g/ml \) (12). Such figures were in general agreement with studies by Carter (4), who used agar plate assays and monaxenic techniques with *Escherichia coli* as the sole source of nutrient. Monaxenic techniques, especially using agar surfaces to cultivate amoebae, have lent themselves poorly to quantitation and to microscopic examination for motility. In addition, the presence of bacteria has added a variable whose

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amphotericin B (MIC ( \mu g/ml ))</th>
<th>Clotrimazole (MIC ( \mu g/ml ))</th>
<th>Miconazole (MIC ( \mu g/ml ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>F-66</td>
<td>0.98</td>
<td>0.62</td>
<td>( \leq 0.15 )</td>
</tr>
<tr>
<td>F-69</td>
<td>1.56</td>
<td>0.39</td>
<td>( &lt;0.19 )</td>
</tr>
<tr>
<td>W-M</td>
<td>1.56</td>
<td>0.78</td>
<td>0.25</td>
</tr>
<tr>
<td>L-L</td>
<td>1.97</td>
<td>1.24</td>
<td>0.39</td>
</tr>
<tr>
<td>T-Y</td>
<td>0.98</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>G-J</td>
<td>1.56</td>
<td>0.62</td>
<td>0.31</td>
</tr>
</tbody>
</table>

*a Numbers represent geometric mean of three experiments.*
effect has been difficult to predict; i.e., too many bacteria may create an anaerobic environment, which may be inhibitory to amoebae, or too few may create a nutrient deficiency problem that may be misinterpreted as a direct inhibitory effect of the drug being tested, or the presence of bacteria or their by-products may possibly degrade or alter the test drugs being examined. However, perhaps most importantly, the presence of bacteria as a nutrient source is not comparable to the bacteria-free environment associated with the diseases produced by these organisms.

In vivo studies in mice also have indicated that amphotericin B administered in therapeutically acceptable quantities is effective in preventing PAM (4, 11). More importantly, however, studies in humans with PAM, from whom the responsible pathogen has been isolated and known to be *Naegleria*, have also indicated that amphotericin B in therapeutic doses may be rapidly effective (24 to 48 h) in sterilizing the central nervous system of infecting amoebae (15). In addition, the only cases of survival (2, 6) from culturally proven cases of PAM have been attributed to amphotericin B, the remarkable aspects of these cases being no apparent sequelae.

The precise mechanism of action of amphotericin B on *Naegleria* is not known; however, as a polyene, amphotericin B binds to sterols in the membrane, preferentially to ergosterol and cholesterol (1). Schuster and Rechthand have recently shown that amphotericin B is amebicidal when used during the lag phase of amebic growth but mainly inhibitory when used during the log phase of growth (24). Ultrastructural changes that they observed were distortions of nuclear shape, increase in cytoplasmic membranes (both rough and smooth endoplasmic reticulum), decrease in food vacuoles, absence of pseudopods, abnormal mitochondria, increase in autophagic vacuoles, and blebbing of the plasma membrane. These investigators also observed that, with increased time of exposure to amphotericin B, these abnormalities increased, a finding supported by our studies, namely, that growth-inhibiting activity was greater after 48 h of exposure to amphotericin B than after 24 h of exposure. Also, by using time-lapse cinematography studies of pathogenic *Naegleria* exposed to lethal concentrations of amphotericin B, we have observed, over a period of less than 1 h, sequential rounding up (loss of pseudopods), increase in number and size of vacuoles (coalescing), development of watery cytoplasm, increased size of organisms, increased visibility of granules, and, eventually, rupturing or disintegration of the trophozoite with complete loss of contents (unpublished data).

It has been suggested in the literature that clotrimazole or some of its analogues might be used to treat PAM due to *Naegleria* (18). Jameson and Anderson, in studying a New Zealand strain of pathogenic *Naegleria*, observed that amebostatic activity of clotrimazole lay in the range of 0.06 to 0.12 μg/ml, and amebicidal activity is in the range of 0.12 to 1.0 μg/ml (18). Although they studied 16 human pathogens and observed a uniform amebicidal concentration of 0.15 μg/ml for an inoculum size of 730 amoebae, they also observed that such a concentration failed to prevent growth when the inoculum size was increased to 7,300 amoebae. Although we also observed that clotrimazole and miconazole inhibited growth of pathogenic *Naegleria* after 24 to 48 h of exposure (Table 2), these agents did so less rapidly and with less predictability than did amphotericin B. For example, in our test system the 24-h growth of W.M., L.L., T.Y., and G.J. was inhibited in low concentrations by both clotrimazole and miconazole, but the two Australian strains (F-66 and F-69) were not. However, after 48 h of exposure to clotrimazole or miconazole, the two Australian strains finally demonstrated susceptibility. Thus, the length of time that was

### Table 3. MMIC and 24- and 48-h MICs of polymyxin B sulfate and pentamidine isethionate against Acanthamoeba

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polymyxin B sulfate*</th>
<th>Pentamidine isethionate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMIC (µg/ml)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td>CH-6</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>CH-5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Garcia</td>
<td>50</td>
<td>79.37</td>
</tr>
<tr>
<td>P6</td>
<td>50</td>
<td>79.37</td>
</tr>
<tr>
<td>Gream</td>
<td>≥100</td>
<td>≥100</td>
</tr>
</tbody>
</table>

* Numbers represent geometric mean of three experiments.  
* Numbers represent geometric mean of only two experiments.
required for inhibition of amoebic growth by both these agents render them of less therapeutic value than amphotericin B. PAM is a disease in which rapid and, if possible, complete effect (less than 24 h) of therapeutic agents used must occur or the patient will probably die or suffer irreversible neurological sequelae. In addition, if the results reported by Jamieson and Anderson (18) of an adverse effect of inoculum size are correct, since studies of the number of amoebae that may be present in the cerebrospinal fluid of infected patients may be in excess of 150,000 trophozoites in the subarachnoid space alone (13), clotrimazole and miconazole would not appear to be indicated to treat this disease.

In general, in vitro studies on motility inhibition (MMIC) of amoebae may be a quick and simple method of suggesting the capability of an agent not only to inhibit the growth of amoebae but, more importantly, to destroy them rapidly (12). Amoebae depend on pseudopodial activity and motility for feeding. Such tests can be performed in any laboratory in 4 to 6 h and can offer the clinician treating the disease valuable guidance early in the course of therapy. By studying motility 6 h after exposure, MMICs of amphotericin B appeared to reliably predict rapid cidal activity and growth inhibition. On the other hand, such studies with miconazole, because of poor motility inhibition, suggested that rapid killing did not occur, even though eventually (by 24 h) inhibition of growth was demonstrated. All other agents tested did not affect motility, and, predictably, they did not inhibit growth.

The lack of or poor susceptibility of pathogenic Acanthamoeba species to all agents tested was disturbing. Only polymyxin B and pentamidine isethionate appeared to be slightly effective, but only in concentrations that would probably be unattainable in the subarachnoid space or brain substance unless introduced intrathecally. Although the two ocular pathogens (Gresham and Garcia) demonstrated the highest resistance, it is possible that in such infections (from which these strains were isolated), ophthalmic solutions could contain concentrations approaching the 24- and 48-h MICs. It is noteworthy that amphotericin B, clotrimazole, and miconazole, although effective against Naegleria, were ineffective against Acanthamoeba. The reason(s) for this is obscure, but for amphotericin B it probably relates to differing concentrations and availability of various sterols and lipids in the membranes of such species.

The lack of susceptibility of Acanthamoeba sp. to 5-fluorocytosine is in contrast to studies by Stevens and O’Dell (25) and Casemore (7).

Their investigations found 5-fluorocytosine to be inhibitory in a range of 12.5 (7) to >40 μg/ml (25); however, Stevens and O’Dell noted that virulent Acanthamoeba sp. were more resistant than avirulent ones (25) and were susceptible only at concentrations exceeding 40 μg/ml. The explanation for resistance of our strains of Acanthamoeba to 5-fluorocytosine is unknown but could have been: (i) due to the enriched media used by us, which probably contained small quantities of competing pyrimidines, e.g., cytosine; (ii) because most of our strains were highly pathogenic and thus inherently more resistant; or (iii) simply due to variations in technique (e.g., solvents, solubility of agents tested, dilutions, media, etc.).

LITERATURE CITED


15. Fowler, M., and R. F. Carter. 1965. Acute pyogenic meningitis probably due to Acanthamoeba sp: a pre-