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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 amoebo-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) encephalitis 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 10^4 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-*

TABLE 1. *Strains of free-living amoebae studied*

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

moeba castellanii. (The Gresham strain was from a human ocular infection [21], courtesy of J. Nagington 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 producing up to 60% mortality in mice inoculated intranasally 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 a series of freshwater isolates collected in the United States by Frederick Page.)

The therapeutic agents tested were as follows: amphotericin B (E.R. Squibb & Sons, Inc., Princeton, 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 (Delbay Pharmaceuticals, Inc., Bloomfield, N.J.) dissolved in dimethyl 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., Nutley, N. J.) dissolved in 0.1 N sodium hydroxide (highest concentration, 0.01%); trimethoprim (Burroughs-Wellcome, Research Triangle Park, N.C.) dissolved in 0.1 N hydrochloric acid (highest concentration, 0.01%); a 20:1 mixture of sulfamethoxazole and trimethoprim; miconazole (Janssen R & D, Inc., New Brunswick, N.J.) plus miconazole placebo (0.115 ml of polyethoxylated castor oil, 0.5 mg of sodium bisulfite, and 1.62 mg of methylparaben per 1 ml); polymyxin B sulfate (Pfizer Laboratories, New York, N.Y.); pentamidine isethionate (Center for Disease Control, Atlanta, Ga.); paramomycin 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 (axenic) in vitro test system was developed and utilized (Fig. 1). Agents to be studied were solubilized in stock solutions from which maximum concentrations 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 culture chamber/slide (Miles Laboratories, no. 4808). Again, using the automatic non-electric pipette, 0.1-ml suspensions containing 10^3 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 10^3 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, American Optical, ordinary light microscope were used.) To one chamber, as appropriate controls, amoebae were added to axenic media, containing, if necessary, 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 trophozoites was generally accompanied by certain morphological changes, as described previously (12): diminution in size, rounding up, watery ectoplasm, increase in granulation, and/or complete disintegration 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 determined. 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.

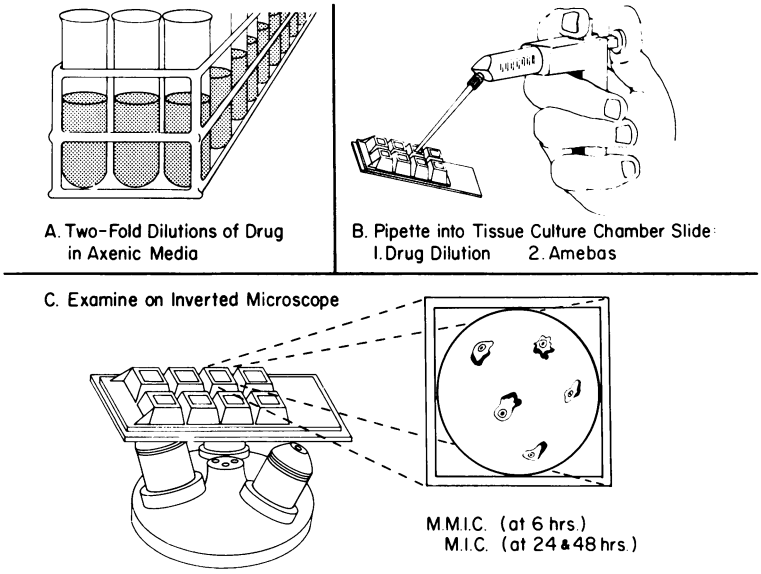

FIG. 1. *Schematic representation of in vitro technique utilized for antiamoebic susceptibility testing (see Materials and Methods for explanation).*

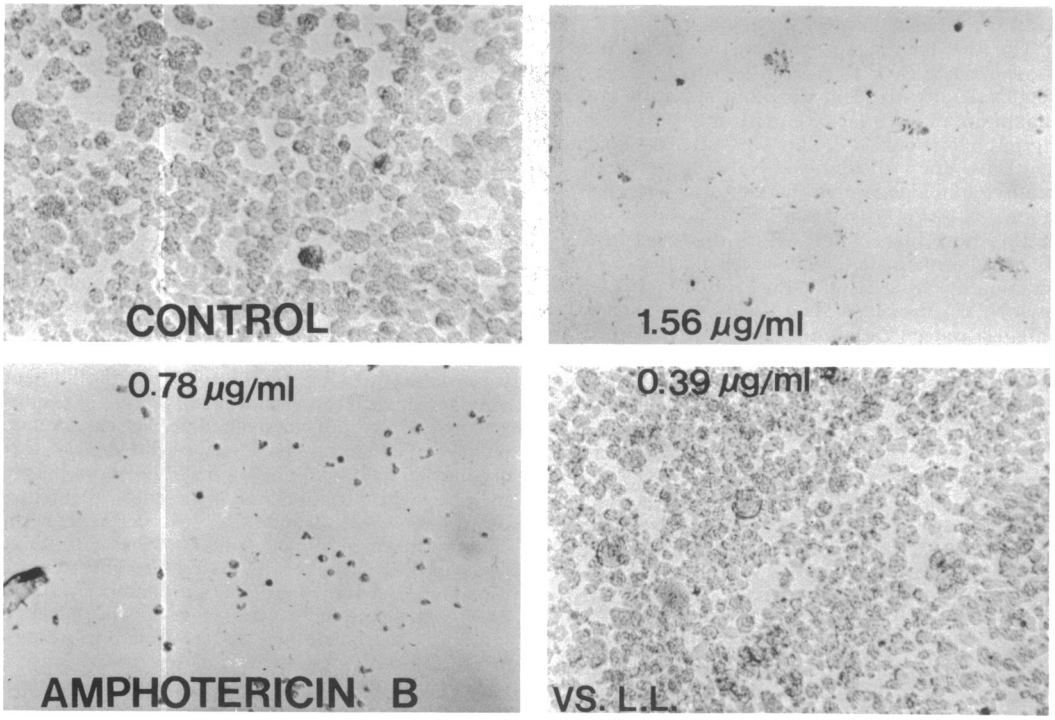

FIG. 2. *Microscopic examination of four chambers containing amphotericin B and a liquid axenic culture of Naegleria. The surface of the control chamber (upper left) is covered with viable, motile amoebae, whereas organisms in a chamber containing 0.39 µg/ml are beginning to round up and lose their motility (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 μg/ml, with a mean MMIC for the group of 1.39 μg/ml, whereas mean MICs at 24 h ranged from 0.39 to 1.24 μg/ml, with a mean MIC for the group of 0.62 μg/ml. Mean MICs of amphotericin B at 48 h against these same six strains ranged from ≤0.15 to 0.39 μg/ml, with a mean for the total of ≤0.26 μg/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 μg/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 ≤0.39 μg/ml to as high as 39.69 μg/ml (Table 2). Similar results were observed for miconazole, as mean MMICs were always above 39.69 μg/ml, whereas mean 24-h MICs ranged from 0.78 to 25 μg/ml. Mean 48-h MICs for both clotrimazole and miconazole fell below those values observed at 24 h, ranging from <0.39 to ≤1.57 μg/ml and 0.98 to 1.97 μg/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 ≥100 μg/ml (mean, ≥60.15) and from 35.36 to ≥70.71 μg/ml (mean, ≥54.27), respectively. MICs of polymyxin B at 24 h were 50 to ≥100 μg/ml (mean, ≥72.36), and at 48 h they were 79.37 to ≥100 μg/ml (mean, ≥87.06). MICs for pentamidine at 24 h ranged from 35.36 to ≥100 μg/ml (mean, ≥45.85), and at 48 h they were from 12.5 to 50 μg/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 μg/ml for the six pathogenic strains tested, and 24- and 48-h MICs ranged from 0.39 to 1.24 and from ≤0.15 to 0.39 μg/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 ≤0.625 μg/ml and was amebicidal at concentrations of ≤0.078 μg/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 2. *MMIC and 24- and 48-h MICs of amphotericin B, clotrimazole, and miconazole against Naegleria*[a]

Strain	Amphotericin B			Clotrimazole			Miconazole		
	MMIC (μg/ml)	MIC (μg/ml)		MMIC (μg/ml)	MIC (μg/ml)		MMIC (μg/ml)	MIC (μg/ml)	
		24 h	48 h		24 h	48 h		24 h	48 h
F-66	0.98	0.62	≤0.15	50	39.69	≤1.57	≥79.37	25	1.97
F-69	1.56	0.39	≤0.19	50	7.94	0.62	≥63	15.74	0.98
W.M.	1.56	0.78	0.25	50	≤0.62	≤0.50	39.69	1.56	1.24
L.L.	1.97	1.24	0.39	50	≤0.39	≤0.50	≥63	1.24	1.24
T.Y.	0.98	0.39	0.39	50	≤0.39	≤0.39	50	0.78	1.24
G.J.	1.56	0.62	0.31	50	≤0.39	≤0.50	≥63	1.56	1.24

[a] Numbers represent geometric mean of three experiments.

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

Strain	Polymyxin B sulfate[a]			Pentamidine isethionate[b]		
	MMIC (μg/ml)	MIC (μg/ml)		MMIC (μg/ml)	MIC (μg/ml)	
		24 h	48 h		24 h	48 h
CH-6	63	63	79.37	≥70.71	≥100	50
CH-5	50	50	79.37	70.71	35.36	12.5
Garcia	50	79.37	≥79.37	35.36	35.36	25
P6	50	79.37	≥100	50	35.36	17.68
Gresham	≥100	≥100	≥100			

[a] Numbers represent geometric mean of three experiments.
[b] Numbers represent geometric mean of only two 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 (13). 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 amoebic 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). Jamieson 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

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 *Acanthamoebae*. 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.).

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