JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1996, p. 385–388 Vol. 34, No. 2
0095-1137/96/$04.00+0

Axenic Growth and Drug Sensitivity Studies of *Balamuthia mandrillaris*, an Agent of Amebic Meningoencephalitis in Humans and Other Animals

F. L. SCHUSTER[1]* AND G. S. VISVESVARA[2]

Department of Biology, Brooklyn College, Brooklyn, New York 11210-2889,[1] and Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30341-3724[2]

Received 17 July 1995/Returned for modification 23 October 1995/Accepted 13 November 1995

A cell-free growth medium for the opportunistic pathogenic ameba *Balamuthia mandrillaris* is presented. This represents an advance over the use of monkey kidney cells for growth of the amebas and can be helpful in isolation of these amebas from brain tissue from cases in which amebic meningoencephalitis is a diagnostic possibility, as well as for biochemical and molecular biological studies. Three isolates of *Balamuthia* have been cultured in this medium. The cell-free growth system was also used to screen cultures for sensitivity to a variety of antimicrobial agents. Of the various drugs tested, pentamidine isethionate was most effective against amebas (ca. 90% inhibition after 6 days of exposure), but the drug was amebastatic and not amebacidal in the axenic system at the highest concentration tested (10 μg/ml).

Balamuthia mandrillaris is an opportunistic ameba that has been identified as an agent of granulomatous amebic meningoencephalitis in humans and animals (6, 7, 9). The ameba has been reported in immunocompromised hosts (1–3, 8), as well as in individuals with no apparent immunologic impairment (4). The organism is different from the free-living pathogenic amebas of the genera *Acanthamoeba* and *Naegleria* (6, 9). Its life cycle consists of a trophic or feeding stage and a dormant cyst stage (10). There have been no reports of isolation of *B. mandrillaris* from the environment, though undoubtedly it occurs in some form, probably as a thick-walled cyst. Both stages have been reported in tissues of infected individuals (6, 9). The organism bears a resemblance to free-living amebas of the genus *Leptomyxa* but has recently been described as a new genus and species because of its unique features (10).

Initial isolates of the ameba were grown on monkey kidney tissue monolayers, which supported growth of the amebas (9). In this report, we describe the axenization of three different isolates of *Balamuthia* in a cell-free culture medium, as well as data on sensitivity of these amebas to a variety of antimicrobial agents. The availability of an axenic growth medium will be helpful in isolation of *B. mandrillaris* from individuals suspected of having granulomatous amebic meningoencephalitis caused by this ameba.

(Portions of this study were presented at the East Coast Regional Meeting on Protozoa held in Raleigh, N.C., in May 1994.)

MATERIALS AND METHODS

Three strains of *B. mandrillaris* were used in this study. The original isolate upon which the description of the ameba as a new genus and species was based (CDC-V039) was from brain tissue of a pregnant mandrill baboon that died at the San Diego Zoo Wild Animal Park (9, 10). A second isolate (CDC-V188) was from the brain of a human from Georgia following an amputation of a leg because of an accident and skin abscess (3). A third isolate was from the brain of a patient with chronic alcoholism from Las Vegas, Nev. (CDC-V194), who had seizures and hemiparesis (9).

Cultivation. Stock cultures of the amebas were maintained on monolayers of African green monkey kidney cells (ATCC CRL 1586) in RPMI 1640 tissue culture medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (Gibco) with 10% fetal or newborn calf serum (Gibco) in Corning tissue culture flasks (25 mm^2) at 37°C. When the amebas had cleared the monolayer by ingesting the tissue culture cells, the flasks were chilled on ice for 5 min to dislodge the amebas. A portion of the supernatant fluid was transferred to a new flask with an already established (1- to 2-day-old) monkey kidney cell monolayer. Depending on the density of the monolayer, the size of the inoculum, and the numbers of amebas per milliliter, it took about 4 to 7 days for the amebas to clear the flask of the monkey kidney cells.

For cultivation of amebas in the axenic medium, trophozoites were collected by centrifugation, counted in a Coulter Counter (model Z_F), and adjusted to give 5,000 amebas per ml in the tissue culture flasks containing 10-ml amounts of the various formulations of growth media (Table 1) at 37°C. Cell counts were taken at intervals of 1 to 2 days for periods of up to 2 weeks, by removing 0.5 ml of culture fluid from chilled flasks.

In preparation of the medium, Biosate peptone, yeast extract, and *Torula* yeast RNA were dissolved in glass-distilled water and autoclaved. Hanks' basal salt solution with calcium and magnesium (Gibco, 10×) was added aseptically after autoclaving to bring the salt concentration to 1×. Supplements, either autoclaved or filter sterilized, were added to complete the medium. Fetal or newborn calf serum (Gibco) was added to media at a concentration of 10%. A serum replacement, CPSR-5 (Sigma) at 10%, was tested in another variation of culture medium. Vitamin 2 mixture (consisting of biotin and folic acid, each at 0.05 mg/100 ml) was used in initial medium formulations but was replaced by minimal essential medium (MEM) vitamin solution (Sigma, 100×). The pH of the medium was adjusted to 7.2 with sterile 1*N* NaOH. Addition of penicillin-streptomycin (Gibco) to the medium is optional if bacterial contamination is a concern.

Drug testing. Antimicrobial agents were tested for activity on trophic *Balamuthia* amebas growing in the presence of monkey kidney cells and in cell-free culture media. For evaluation of drug effects on amebas growing on tissue culture cells, monkey kidney cell cultures were established in tissue culture petri plates (35 mm) or 12-well tissue culture plates, containing 3 ml of medium (RPMI 1640 with HEPES buffering plus newborn calf serum), and incubated at 37°C in a 5% CO_2 atmosphere. When the kidney cells formed a monolayer across the plate surface, supernatant growth medium was aspirated and replaced with fresh RPMI 1640 medium, and amebas were added at a concentration of 2,000 amebas per ml. A period of ca. 24 h was allowed to elapse to give amebas time to settle and start feeding on the tissue culture cells. At that time, antimicrobial agents were added to the dishes from stock solutions of 2 mg/ml to give final concentrations of 1 and 10 μg/ml. Control plates were used to evaluate toxicity of the antimicrobial concentration for the tissue culture cells and to evaluate monolayer destruction in the absence of the antimicrobial agent. Growth of amebas, as determined by further destruction of the kidney cell monolayer, was evaluated subjectively on days 6 to 7 by examination of the dishes with an inverted tissue culture microscope. As amebas proliferated in the monolayer, they ingested and destroyed the tissue culture cells. In several experiments in which ameba growth

* Corresponding author. Mailing address: Department of Biology, Brooklyn College, 2900 Bedford Ave., Brooklyn, NY 11210. Phone: (718) 951-5711.

TABLE 1. *Balamuthia mandrillaris* medium (BM-3)

Process and component (supplier)	Amt
Autoclaved	
Biosate peptone (BBL)	2.0 g
Yeast extract (Difco)	2.0 g
Torula yeast RNA (Sigma)	0.5 g
Glass-distilled water	345.0 ml
Added aseptically to above after autoclaving	
Hanks' balanced salts, 10× (Gibco)[a]	34.0 ml
5% ox liver digest in Hanks' salts (Panmede)[b]	100.0 ml
MEM vitamin mixture, 100× (Sigma)[a]	5.0 ml
Lipid mixture, 1,000× (Sigma)[a]	0.5 ml
MEM nonessential amino acids, 100× (Sigma)[a]	5.0 ml
10% glucose[c]	5.0 ml
Hemin at 2 mg/ml (Mann Research)[c]	0.5 ml
0.5% taurine (Sigma)[d]	5.0 ml
pH adjusted to 7.2 with sterile 1*N* NaOH	
Added at time of use	
Newborn calf serum (Gibco) to give 10%[a]	

[a] Sterile from supplier.
[b] Autoclaved in distilled water; Hanks' salts (10×) added upon cooling.
[c] Autoclaved.
[d] Filter sterilized.

was halted as indicated by an intact tissue culture monolayer, antimicrobial agent-containing medium was aspirated from the plate and replaced by fresh medium, and the plates were observed over several days for destruction of the tissue culture monolayer (an amebastatic effect of the antimicrobial agent) or the absence of destruction (an amebacidal effect of the antimicrobial agent).

For evaluation of drug effect on amebas growing in axenic media, cultures were set up in tissue culture flasks with 10 ml of medium containing 5,000 amebas per ml, and drugs were added to give concentrations of 1 and 10 μg/ml. Samples (0.5 ml) for counting on the Coulter Counter were removed at intervals of 1 to 2 days from flasks chilled to loosen ameba attachment to the flask surface. Growth was observed for about a week. At the end of several experiments, any remaining amebas were harvested, washed free of antimicrobial agent-containing medium, and resuspended in fresh medium to determine whether the drug was amebastatic or amebacidal. Antimicrobial agents tested included amphotericin B (as Fungizone [Gibco]) and amphotericin methyl ester (E. R. Squibb); the azole compounds bifonazole, clotrimazole (Cutter Biological), ketoconazole (Janssen), and fluconazole (Pfizer); the macrolides azithromycin and clarithromycin (Abbott Laboratories); the amidines pentamidine isethionate (May & Baker) and propamidine isethionate (Rhone-Poulenc Rorer); trimethoprim and sulfamethoxazole (Sigma), each alone and in a 1:5 ratio; and gramicidin S and polymyxin B (Sigma).

RESULTS AND DISCUSSION

Although little more than a curiosity when first isolated, *Balamuthia* amebas have been identified from an increasingly large number of cases of amebic meningoencephalitis in humans and other animals (1–4, 6–10). Indeed, cases of amebic meningoencephalitis originally thought to have been caused by *Acanthamoeba* spp. have now been identified as having been caused by *B. mandrillaris* on the basis of the use of the indirect immunofluorescence technique applied to brain tissue (9). Since *Balamuthia* amebas form cysts in brain tissue, it has been possible to recover these amebas from frozen brain sections obtained upon autopsy. In earlier isolations, amebas appeared and grew out when minced brain tissue was added to tissue culture monolayers. This, however, requires the availability of tissue culture cells and facilities for maintaining them. The availability of a readily prepared cell-free growth medium provides a means of isolating this ameba from brain tissues of individuals in which a diagnosis of amebic meningoencephalitis is suspected and can serve to corroborate evidence from indirect immunofluorescence studies. Thus, BM-3 medium is suggested as an effective growth medium for isolation and cultivation of these opportunistic pathogens and provides the basis for biochemical and molecular characterization of this unusual group of amebas.

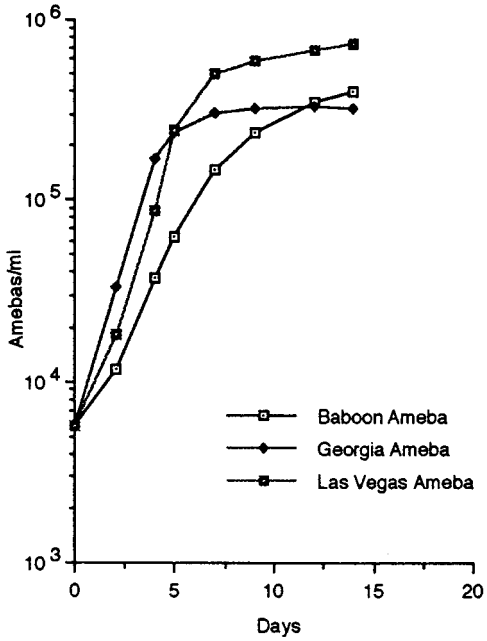

FIG. 1. Growth of three *Balamuthia* isolates in BM-3 medium at 37°C.

Growth. *Balamuthia* amebas from monkey kidney cell cultures were harvested, washed in basal RPMI 1640 (without serum), and suspended in different variations of cell-free growth media. The first of these variations (BM-1) consisted of RPMI 1640 medium supplemented with peptone, yeast extract, *Torula* yeast RNA, vitamin 2 mixture, hemin, and newborn calf serum to 10%. In the second variation (BM-2), RPMI 1640 was omitted, with the fluid volume made up by Hanks' basal salts at 1×, and glucose was added. In variation BM-3, the current variation (Table 1), MEM vitamin solution replaced vitamin 2 mixture, and MEM nonessential amino acid solution, lipid mixture, and taurine were added to the growth medium. Ameba growth increased with each variation in medium composition. The increase in cell numbers from BM-1 to BM-3 was

TABLE 2. Sensitivity of *Balamuthia mandrillaris* to antimicrobial agents[a]

Drug	1 μg/ml	10 μg/ml
Azithromycin	0	29
Clarithromycin	0	24
Bifonazole	67	
Clotrimazole	34	
Fluconazole	0	34
Ketoconazole	0	80
Pentamidine isethionate	82	93
Propamidine isethionate	80	93
Gramicidin S	0	97
Polymyxin B	49	96
Amphotericin B	19	47
Amphotericin B methyl ester	0	6
Trimethoprim	0	0
Sulfamethoxazole	0	0
Trimethoprim-sulfamethoxazole (1:5)	0	0

[a] Data are presented as the percent inhibition of ameba growth scored on day 6.

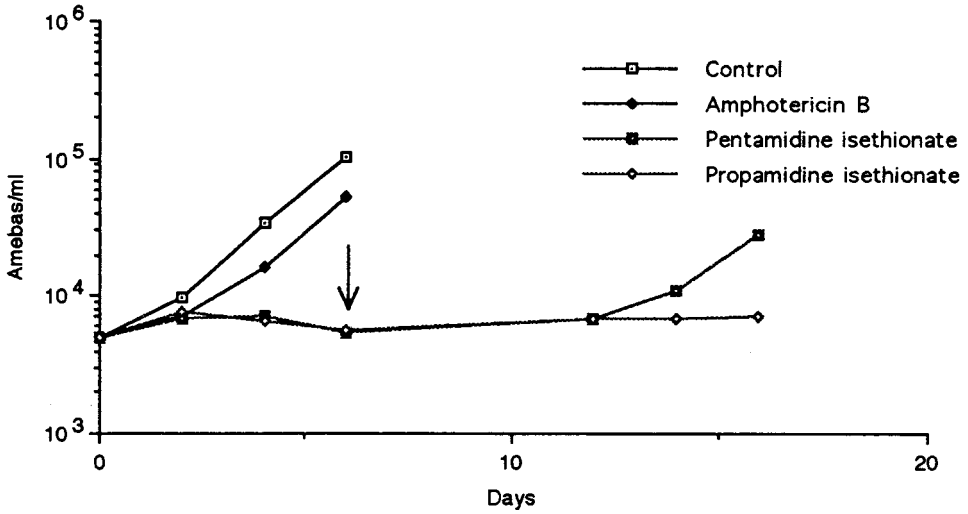

FIG. 2. Inhibition of *B. mandrillaris*, the baboon isolate, in three antimicrobial agents at 10 μg/ml in axenic medium. In these experiments, the amebas were grown in the drug for 7 days, washed free of the drug at day 7 (arrow), and resuspended in fresh medium. Resumption of ameba growth occurred after pentamidine treatment but only after 5 days (amebastatic effect). No such revival of amebas occurred following propamidine treatment (amebacidal effect).

about 75% and from BM-2 to BM-3 was about 30%. This was probably due in part to an increase in nutrient levels, but also to selection of amebas for growth in the media. In BM-3, the baboon and Georgia isolates of *B. mandrillaris* gave ca. 350,000 amebas per ml after 12 days in culture; the Las Vegas isolate gave ca. 675,000 amebas per ml after 12 days in culture (Fig. 1). Doubling times were 28, 20, and 21 h for the baboon, Georgia, and Las Vegas isolates, respectively.

Several additives such as the vitamin solution, lipid mixture, and nonessential amino acid solution had a beneficial effect upon growth and can be commercially obtained for ease of medium preparation. The use of these components and taurine was based on their inclusion in a medium developed by Kleinschuster and Swink (5) for a protozoal oyster pathogen. Other liver preparations (for example, Bacto, Oxoid, and Sigma liver concentrates) can be substituted for Panmede ox liver, which is no longer commercially available. The serum replacement CPSR-5, containing bovine embryonic fluid, has been used in place of newborn calf serum, with no difference in cell numbers produced.

Amebas in this growth medium were in the trophic stage. As the cultures aged, trophic amebas began to encyst. Encystation, however, occurred in only a small fraction of the population, with most of the amebas remaining as trophozoites. Growth was better at 37°C than at 30°C, though amebas grew even at 25°C. Active trophozoites can be found in cultures at all temperatures even after several months; the amebas, however, become smaller in size.

Drug sensitivity. In the ameba-tissue culture system, amphotericin B, pentamidine isethionate, and propamidine isethionate suppressed ameba growth at a concentration of 1 μg/ml and prevented destruction of the monkey kidney cell monolayer. Amebas recovered after about 3 days when the amphotericin-containing medium was aspirated from tissue culture plates and replaced with fresh RPMI 1640 medium, suggesting that the drug was amebastatic. No recovery occurred when the pentamidine and propamidine-containing media were replaced with fresh tissue culture medium. Thus in the ameba-tissue culture system, these two drugs appeared to be amebacidal. Amphotericin B methyl ester, clotrimazole, and ketoconazole were ineffective in blocking ameba growth. Propamidine isethionate was toxic for monkey kidney cells at both 1 and 10 μg/ml, and amphotericin B showed toxicity at 10 μg/ml.

The effects of antimicrobial agents on *Balamuthia* amebas growing in cell-free growth medium (BM-2 and BM-3) are presented in Table 2. Results of testing are presented as percent inhibition of cell growth compared with control cultures run at the same time. Antimicrobial agents with little or no effect included azithromycin and clarithromycin, the azole compounds, the antimetabolites trimethoprim and sulfamethoxazole, and amphotericin B methyl ester. Amphotericin B was marginal in its effects. The most effective antimicrobial agents were pentamidine and propamidine isethionates, polymyxin B, and gramicidin S. Recovery from pentamidine or polymyxin B inhibition occurred after 7 to 9 days, when the antimicrobial agent-containing media were replaced with fresh medium. Thus, these antimicrobial agents were amebastatic at the concentrations tested. Amebas treated with propamidine or gramicidin S did not recover after 7 to 10 days, suggesting an amebacidal effect of these drugs. Figure 2 presents data on growth inhibition and recovery for amphotericin B, pentamidine, and propamidine. Although propamidine, gramicidin, and polymyxin appeared to be amebacidal, these antimicrobial agents are not suitable for parenteral use. Pentamidine isethionate, though it is amebastatic in the cell-free test system, appears to be the best choice as a potential antimicrobial agent for treatment of amebic meningoencephalitis caused by *B. mandrillaris*.

REFERENCES

1. **Anzil, A. P., C. Rao, M. A. Wrzolek, G. S. Visvesvara, J. H. Sher, and P. B. Kozlowski.** 1991. Amebic meningoencephalitis in a patient with AIDS caused by a newly recognized opportunistic pathogen. Arch. Pathol. Lab. Med. **115:**21–25.
2. **Chimelli, L., M. D. Hahn, F. Scaravilli, S. Wallace, and G. S. Visvesvara.** 1992. Granulomatous amoebic encephalitis due to leptomyxid amoebae: report of the first Brazilian case. Trans. R. Soc. Trop. Med. Hyg. **86:**635.
3. **Gordon, S. M., J. P. Steinberg, M. H. DuPuis, P. E. Kozarsky, J. F. Nickerson, and G. S. Visvesvara.** 1992. Culture isolation of *Acanthamoeba* species and leptomyxid amebas from patients with amebic meningoencephalitis, including two patients with AIDS. Clin. Infect. Dis. **15:**1024–1030.
4. **Griesemer, D. A., L. L. Barton, C. M. Reese, P. C. Johnson, J. A. B. Gabrielsen, D. Talwar, and G. S. Visvesvara.** 1994. Amebic meningoencephalitis caused by *Balamuthia mandrillaris*. Pediatr. Neurol. **10:**249–254.

5. **Kleinschuster, S. J., and S. L. Swink.** 1993. A simple method for the *in vitro* culture of *Perkinsus marinus*. Nautilus **107:**76–78.
6. **Martinez, A. J., A. E. Guerra, J. Garcia-Tomayo, G. Cespedes, J. E. Gonzalez-Alfonso, and G. S. Visvesvara.** 1994. Granulomatous amebic encephalitis: a review and report of a spontaneous case from Venezuela. Acta Neuropathol. **87:**430–434.
7. **Neafie, R. C., and A. M. Marty.** 1993. Unusual infections in humans. Clin. Microbiol. Rev. **6:**34–56.
8. **Taratuto, A. L., J. Monges, J. C. Acefe, F. Meli, A. Parades, and A. J. Martinez.** 1991. Leptomyxid amoeba encephalitis: report of the first case in Argentina. Trans. R. Soc. Trop. Med. Hyg. **85:**77.
9. **Visvesvara, G. S., A. J. Martinez, F. L. Schuster, G. J. Leitch, S. V. Wallace, T. K. Sawyer, and M. Anderson.** 1990. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. J. Clin. Microbiol. **28:** 2750–2756.
10. **Visvesvara, G. S., F. L. Schuster, and A. J. Martinez.** 1993. *Balamuthia mandrillaris*, n. g., n. sp., agent of amebic meningoencephalitis in humans and other animals. J. Eukaryot. Microbiol. **40:**504–514.