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Balamuthia mandrillaris from soil samples

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Balamuthia mandrillaris amoebas are recognized as a causative agent of granulomatous amoebic encephalitis, a disease that is usually fatal. They were first recognized when isolated from the brain of a mandrill baboon that died in the San Diego Zoo Wild Life Animal Park. Subsequently, the amoebas have been found in a variety of animals, including humans (young and old, immunocompromised and immunocompetent persons), in countries around the world. Until recently, the amoebas had not been recovered from the environment and their free-living status was in question. The recovery of a *Balamuthia* amoeba from a soil sample taken from a plant at the home of a child from California, USA, who died of *Balamuthia* amoebic encephalitis, was reported previously. In a continued investigation, a second amoeba was isolated from soil that was obtained from an outdoor potted plant in a spatially unrelated location. A comparison of these two environmental amoebas that were isolated from different soils with the amoeba that was obtained from the child's clinical specimen is reported here. Included are the isolation procedure for the amoebas, their growth requirements, their immunological response to anti-*Balamuthia* serum, their sensitivity to a selection of antimicrobials and sequence analysis of their 16S rRNA gene. The evidence is consistent that the amoebas isolated from both soil samples and the clinical isolate obtained from the Californian child are *B. mandrillaris*.

INTRODUCTION

The death of a pregnant mandrill baboon in the San Diego Zoo Wild Life Animal Park from meningoencephalitis led to the isolation of an amoeba from macerated necrotic brain tissue (Visvesvara *et al.*, 1990). The amoebas failed to grow on agar with a bacterial overlay, which is the usual technique for isolation of pathogenic amoebas from brain tissue; they were later cultivated on monkey kidney cells (MKCs) (Visvesvara *et al.*, 1990). Because of similar morphologies of the trophic and cystic stages, the new isolate was at first grouped with the leptomyxid amoebas (Anzil *et al.*, 1991) and later described as a new genus and species, *Balamuthia mandrillaris* (Visvesvara *et al.*, 1993). Once in culture, antibodies against the new isolate were produced and used for indirect immunofluorescent testing and comparison with other brain-tissue samples. Retrospectively, some human amoebic encephalitis cases in which the causative agent was either unidentified or described as *Acanthamoeba* were found to be related immunologically to the baboon isolate (Visvesvara *et al.*, 1993). *Balamuthia*, thus, joined members of the genus *Acanthamoeba* and *Naegleria fowleri* as free-living, pathogenic amoebas (Martinez, 1985). The free-living status of *Balamuthia* was open to question, as it had never been isolated from the environment like the other pathogenic amoebas, but only from clinical specimens (Schuster & Visvesvara, 1996).

Balamuthia encephalitis is an insidious disease with an incubation period that can range from days to as long as 2 years (Rowen *et al.*, 1995). Likely routes of infection are through wounds in the skin that become contaminated by soil or through the lower respiratory tract by inhalation of cysts carried by wind-blown soil (Martinez & Visvesvara, 1997). Symptoms include general malaise, headache, fever and neurological involvement (Rowen *et al.*, 1995). Over 100 human cases from geographical regions around the globe have been identified. Infections have developed in both immunocompromised and immunocompetent persons, ranging in age from 4 months to 72 years (Rowen *et al.*, 1995). Because of the absence of specific symptoms, most *Balamuthia* encephalitis cases have been diagnosed post-mortem. Infections have been identified by demonstration of amoebas in biopsy or necropsy tissues, by isolation and growth of the amoebas in culture or by detection of *Balamuthia* antibodies in patients' sera by indirect immunostaining (Schuster *et al.*, 2001).

The death of a young child in California, USA, from *Balamuthia* encephalitis in the spring of 2001 (Bakardjiev

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain OK1 determined in this study are AY648576 (5′ section) and AY648577 (3′ section).

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et al., 2003) provided an opportunity to attempt to isolate *Balamuthia* amoebas from the child's environment. A soil sample from a flowerpot in the child's home was the source of the first environmental isolation of the amoeba (RP5; Schuster *et al.*, 2003). We report here the isolation of a second environmental *Balamuthia* strain (OK1) from soil samples that were taken from a Californian location that was distant and unrelated to the previous soil collection. We describe its isolation procedure and compare its general morphology, growth conditions, immunological response, antimicrobial sensitivity and 16S rRNA gene sequence with those of the clinical isolate from the child (SAm) and the environmental isolate, RP5, from the child's home.

METHODS

Amoebal isolation. Soil samples (5–15 g) from four unrelated outdoor potted plants were collected in sterile vials. The dry soil was hydrated with sterile water (5–20 ml) and one to two drops of the suspension were placed onto individual 100 mm Petri plates containing 1·5 % non-nutrient agar coated with *Escherichia coli*, a standard procedure for the isolation and growth of soil amoebas (Page, 1988; Schuster *et al.*, 2003). Incubation was at room temperature (approx. 20 °C). Plates were kept sealed in plastic bags to prevent drying and examined with an inverted microscope at 63–100 × magnification. Following the detection of large, multipseudopodial amoebas (OK1) feeding on other soil organisms in and under the agar, portions of the agar that contained these amoebas were excised and transferred onto new bacteria-coated plates. After the amoebas of interest had migrated away from fungal and other contaminants, they were then transferred in agar cores to fresh, bacteria-coated agar plates. Between 10 and 20 transfer steps were necessary to separate the OK1 amoebas from most other contaminating organisms in the soil. Fungizone, an antifungal substance, was not used routinely, as its effects on amoebal growth were unknown. With the aim of growing the *Balamuthia*-like amoebas with cultured animal cells, it was important to reduce the bacterial load in the agar pieces containing the amoebas of interest and, at the same time, to provide them with a food source. To accomplish this, transfers of the amoebas in fungus-free agar pieces were made onto agar plates containing *Naegleria gruberi* in a thin film of axenic medium (Balamuth, 1964). Pieces of agar containing the OK1 amoebas were transferred to MKCs in culture flasks (25 cm^2) in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum and penicillin/streptomycin (200 U ml^{-1}) and incubated at 37 °C in a 5 % CO_2 atmosphere. The accompanying *Naegleria* amoebas did not survive at the elevated temperature; however, other small amoebas from the soil sample did and they, like OK1, fed on the MKCs. These other amoebas were eliminated from the OK1 culture by cloning.

Clonal isolation of the amoebas. To separate the *Balamuthia*-like amoebas from the accompanying small soil amoebas, the medium from MKC cultures supporting the growth of both was adjusted to a near-end-point dilution of the amoebas. Aliquots (1 µl) were placed onto the bottom of each well of a 24-well plate. After inspection of each drop with an inverted microscope at 100 ×, those wells that contained only one large amoebic trophozoite or one large amoeba cyst were marked; freshly trypsinized MKCs were added to each marked well and incubation was continued at 37 °C. After 7–10 days, in some of the wells, a single, plaque-like area of destroyed MKCs in the cell sheet contained large trophozoites. No small amoebas were present. Within a few additional days, numbers of the large, trophic amoebas had increased; they were concentrated at the rim of the enlarging plaque and others were floating free in the medium. Amoebas in such cultures were considered to be clones. Representatives of the amoebas emanating from a trophozoite or from a cyst were transferred into culture flasks of MKCs at 37 °C for expanded growth. Amoebas from the SAm and RP5 cultures were likewise cloned and used for comparative study with the new OK1 isolates.

An attempt was made to grow the isolated OK1 amoebas in axenic BM3 medium (Schuster & Visvesvara, 1996). The medium in the flasks was replaced at weekly intervals with DMEM containing first 50, then 75 and finally 100 % BM3 medium.

Antimicrobial sensitivity. All three amoebas were tested for sensitivity to six antimicrobials as described previously (Schuster & Visvesvara, 1996): amphotericin B (as Fungizone), azithromycin, fluconazole, 5-fluorocytosine (flucytosine), pentamidine isethionate and sulfadiazine, at concentrations of 1, 5 and 10 µg ml^{-1}.

Serology. The new isolate, OK1, was tested with both rabbit and human anti-*Balamuthia* sera in order to verify that it was in fact *Balamuthia* by using methods described previously (Schuster *et al.*, 2003). The human serum was from the child who died of the *Balamuthia* infection and had an anti-*Balamuthia* antibody titre of 1 : 256 when tested against *Balamuthia* amoebas from culture by indirect immunofluorescent staining. Testing was done on the SAm and RP5 isolates for comparison. Rabbit anti-*Acanthamoeba* serum was also tested, in order to eliminate the possibility that the isolate might be *Acanthamoeba* and to determine whether any cross-reactivity occurred between the two different amoebas.

DNA extraction, purification and amplification. Cultures of the OK1 isolate were grown on MKCs in tissue-culture medium at 37 °C until the amoebas had cleared the flask of tissue-culture cells. Flasks were chilled and then shaken gently to release the amoebas from the growth surface. Amoebas were pelleted by centrifugation and lysis buffer was added (Casas *et al.*, 1995). The suspension was vortexed and allowed to remain at room temperature for 10 min. 2-Propanol was added to precipitate the nucleic acid, the tubes were vortexed again and then centrifuged for 10 minutes at 10 000 ***g***. The supernatant was aspirated and the pellet was washed with 0·75 ml 70 % ethanol, vortexed and then centrifuged. Alcohol was removed and the tubes were placed in a heating block at 65 °C to drive off residual alcohol, after which they were cooled to room temperature and stored frozen.

PCR amplification was done with the primer set 5′ Balspec16S and 3′ Balspec16S (Booton *et al.*, 2003b), which amplifies a 1075 bp portion of the mitochondrial 16S rRNA gene of *B. mandrillaris*. The PCR product was run on a 1 % agarose gel and purified with a Prep-A-Gene purification kit (Bio-Rad). The concentration of gel-purified DNA was determined by using Low Mass DNA ladder (Invitrogen). The final elution volume was 50 µl.

DNA sequencing. PCR products amplified with the primer pair (see above) were sequenced with the amplification primers 5′ Balspec16S (5′-CGCATGTATGAAGAAGACCA-3′) and 3′ Balspec16S (5′-TTACCTATATAATTGTCGATACCA-3′) and the internal primer mt900 (5′-CAAATTAAACCACATACT-3′), which determines the phylogenetically informative 5′ region of the amplimer (Ledee *et al.*, 2003). Fluorescent sequencing was done on an ABI 310 automated sequencing system (Applied Biosystems) by using an ABI BigDye sequencing kit (version 2.0), following the manufacturer's protocol.

Phylogenetic analysis. Mitochondrial 16S rRNA gene sequences obtained from fluorescent cycle sequencing were aligned to the 16S

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rRNA gene sequence of *B. mandrillaris* and other mitochondrial 16S rRNA gene sequences with the sequence alignment program ESEE (Cabot & Beckenbach, 1989).

RESULTS

Amoebal isolation

Periodic microscopic examination of the bacteria-coated agar plates containing soil from each of the four samples showed a rapid outgrowth of small amoebas, numerous ciliates, nematodes and fungi. After a period of 2–4 weeks, one of the plates contained large *Balamuthia*-like amoebas with numerous protruding pseudopodia beneath the agar surface and among the fungal hyphae (OK1; Fig. 1a). The isolation procedure was repeated for each of the four samples. Again, the *Balamuthia*-like amoebas were present in the same sample; none were found in the other three. The large amoebas were similar in appearance and location within and under the agar to the *Balamuthia* amoeba that was isolated from the home of the child who died of encephalitis (RP5; Fig. 1c).

During the course of isolation, the OK1 amoebas were

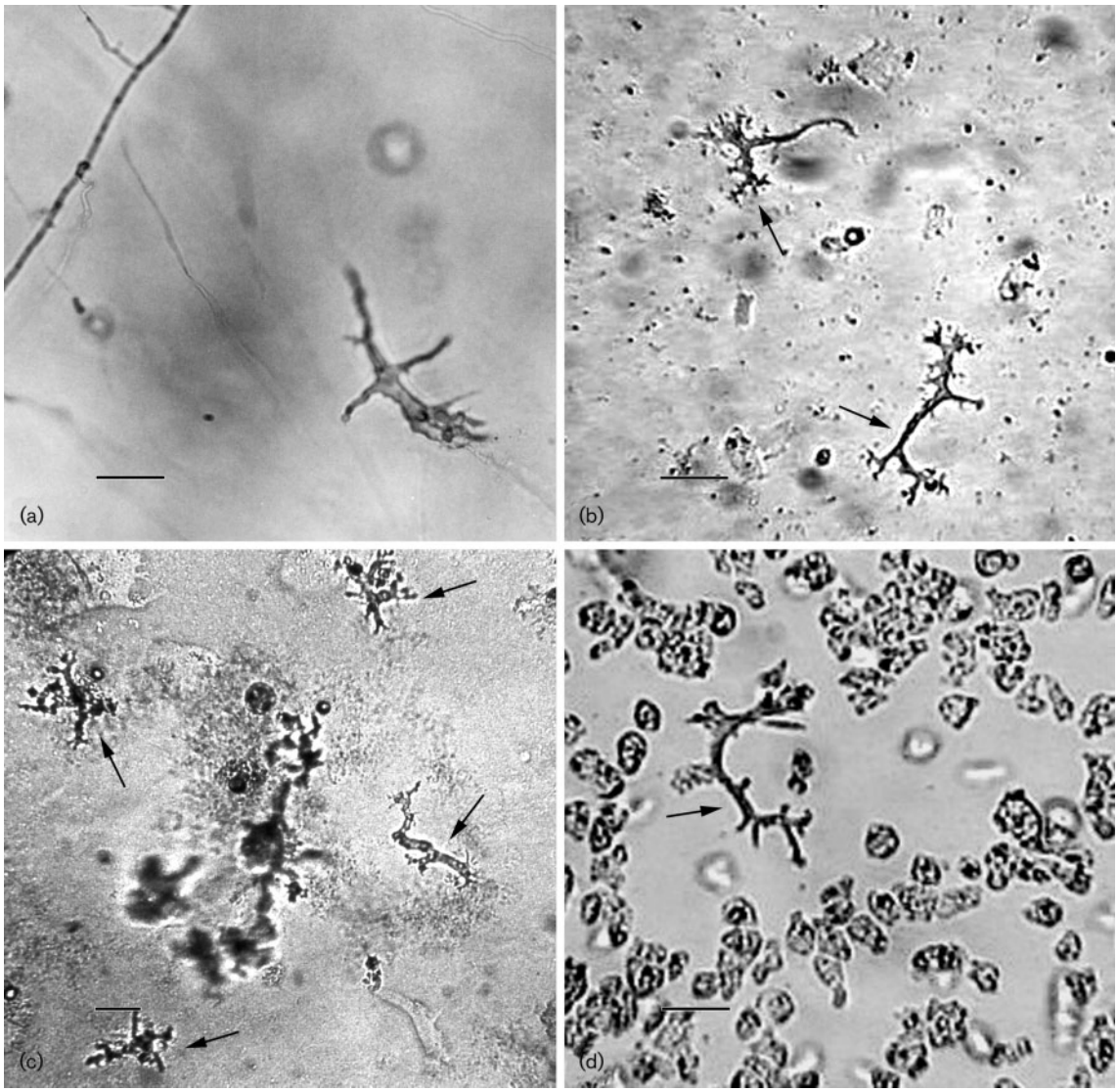

Fig. 1. Amoebas obtained from soil samples. (a) OK1 amoeba shown within the agar, near fungal hyphae. The out-of-focus cyst on the surface indicates the depth of the amoeba in the agar (room temperature). (b) OK1 amoebas (arrows) present with the residual debris after feeding on and destroying cultured MKCs (37 °C). (c) RP5 amoebas (arrows) within the agar at different focus levels with other organisms and debris from the soil sample (room temperature). (d) A soil amoeba (DY) from a yard near a drain. It is larger than the RP5 and OK1 isolates, although similar in morphology and growth conditions; it is separated from other soil organisms and feeding on abundant *N. gruberi* on an agar surface (room temperature). Original magnification, 300 ×. Bars, 20 μm.

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separated from fungal growth by selectively passaging portions of agar in which the amoebas had moved away. In the course of this procedure, most of the other soil organisms were also eliminated. However, the excised pieces of agar containing OK1 also carried bacteria that, despite the presence of antibiotics, overwhelmed MKC cultures if transplanted directly. An intermediate step of adding the OK1 agar explants to agar that had been coated with *Naegleria* amoebas cultivated in axenic medium provided food for the OK1 amoebas and reduced the bacterial concentration sufficiently that subsequent transfers onto monolayers of MKCs were successful. The *Naegleria* amoebas that were also in the agar did not survive at the MKC incubation temperature. An additional problem arose when, in some of the MKC cultures that received OK1 agar explants, small amoebas, presumably from the soil, also grew and destroyed the MKCs. The OK1 amoebas survived in those cultures and could be separated from the small amoebas by cloning. Individual OK1 trophozoites or cysts that were placed into wells in culture plates multiplied as the MKCs that had been added to them were destroyed. The amoebas were first seen as a few trophozoites in a small clearing between the MKCs. Within a week or so, the clearing enlarged as the amoebas at the 'feeding edge' eventually consumed the cell sheet. Individual amoebas appeared to be scattered in the centre of the enlarging plaque or 'floating' in the medium. The OK1 amoebas formed a dense front as they fed at the rim of plaques in the MKCs, after which they remained scattered at some distance from each other as they moved among the debris of the cleared culture (Fig. 1b).

Cloned populations originating from the 113 OK1 amoebas and cysts isolated resulted in a yield of 36 %; the number of clonal cultures was divided equally between those resulting from trophozoites or from cysts. Amoebal clones that were selected for expanded growth exhibited a similar morphology and growth pattern.

Morphology

The general morphology of the amoebas from the two environmental soil isolates and that from clinical material was very similar. The trophozoites varied considerably in shape and their length was in the range of 30–120 μm. Likewise, cysts of the three amoebas had a thick wall and were similar in size (Visvesvara *et al.*, 1990).

In vitro growth

Although both SAm and RP5 amoebas were established in BM3 axenic medium, the new OK1 isolate did not grow and could not be maintained in a cell-free medium. The OK1 amoebas, however, grew well with MKCs at 37 °C and are presently being cultured with tissue cells.

Serology

The immunostaining reactions of the amoebas against rabbit anti-*Balamuthia* serum, measured at twofold dilution steps, showed that the OK1 amoebas (titre of 1 : 128) were somewhat less sensitive than the RP5 (titre of 1 : 512) and the SAm (titre of 1 : 256) amoebas. The amoebas also stained with human anti-*Balamuthia* serum. Thus, the response to antisera showed a positive identity with *B. mandrillaris* and ruled out the possibility that the isolates could be *Acanthamoeba*.

Antimicrobial sensitivity

Pentamidine isethionate was very effective at preventing the growth of all three amoebas at concentrations of 1, 5 and 10 μg ml^{-1}. Little or no inhibition of amoebal growth was found after treatment with fluconazole, 5-fluorocytosine or sulfadiazine. The response to amphotericin B gave variable results in different experiments. Azithromycin did not inhibit growth of OK1, but was generally effective against SAm and RP5. With the exception of the azithromycin results, the three amoebas had similar antimicrobial profiles. The distinction of the antimicrobial effect as being amoebastatic or amoebicidal was not determined.

DNA sequence analysis

Amplification and sequencing of the mitochondrial 16S rRNA gene from OK1 showed that it was identical to those of the two other Californian isolates, SAm and RP5. By using a primer pair that was specific for the 16S rRNA gene, an amplimer of 1075 bp was produced, similar to the amplimers that were produced from the other *Balamuthia* isolates (Booton *et al.*, 2003b).

DISCUSSION

A second environmental isolate of an amoeba was found to be similar to the other Californian environmental isolate, except for resistance to growth in cell-free medium and lack of sensitivity to the macrolide antimicrobial azithromycin. Based on morphology, serology and DNA sequence analysis, the new isolate was determined to be *B. mandrillaris*. Finding a second environmental isolate of *Balamuthia* confirms that they are free-living members of the protozoal soil ecosytem. Despite interest over several decades in the isolation of pathogenic, free-living amoebas, *Balamuthia* amoebas have eluded recognition in soil samples. The standard technique for isolation from soil uses bacteria as the food source for the amoeba and the cultures are observed for a relatively short time (Page, 1988). *Balamuthia*, however, feeds on other amoebas, not bacteria. By comparison, they grow slowly, taking weeks before the amoebas are recognized microscopically amongst other amoebas, ciliates, worms and fungi. Their separation from other soil inhabitants is labour-intensive and involves teasing apart portions of the agar that contain them and culturing them on fresh medium. The use of the small amoeba *N. gruberi* to serve as an intermediate food source helps to reduce an overwhelming load of bacteria prior to the growth of *Balamuthia in vitro* under conditions that

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are not favourable to *Naegleria*. Inclusion of cloning steps provides a uniform culture of amoebas for further study. With knowledge that *Balamuthia* can be recovered from soil, it would be desirable to study soils from other locations to determine the ecological distribution of these amoebas and the potential risk that they present as disease agents for humans and animals.

It is of interest to note that the isolation of two environmental *Balamuthia* strains was from soil in potted plants. Such soil is often enriched organically with additives (chicken manure, earthworm castings, bat guano etc.), making it a rich environment for bacterial growth and, through the food chain, for organisms that feed on bacteria and one another. The recovery of these amoebas from the soil of potted plants may have been fortuitous, in that their population density in that rich environment, along with the heavy yield of other soil organisms, was sufficient to yield growth from the small aliquot of one to two drops of soil suspension that was added to the agar.

Soil has also been a factor in two cases of *Balamuthia* encephalitis that occurred in immunocompetent individuals. In one case, a Californian man working in his backyard developed an infection soon after sustaining a puncture wound that was probably contaminated by soil (Deetz *et al.*, 2003) and, in a second case, a woman from New York was reported to have worked in her garden with compost soil prior to developing an infection (Jung *et al.*, 2004). Based on these two cases and others that have developed around the world, it appears that *Balamuthia* amoebas are not confined to enriched soils, but may be dispersed widely in a variety of soil ecosystems. Two additional *Balamuthia*-like amoebas that are in the process of being isolated are similar in morphology and growth requirements to the OK1 isolate that is described in this paper. One was from garden soil that had not been fertilized for many years and the other, larger in size than the other environmental isolates, was from soil near a backyard shower drain (strain DY; Fig. 1d).

Analysis of the mitochondrial 16S rRNA genes from OK1 and the other environmental and clinical isolates shows sequence similarity to all *Balamuthia* isolates that have been established in culture (Booton *et al.*, 2003a). Sequence variation in the 16S rRNA genes of all *Balamuthia* isolates ranges from 0 to 1·8 %, compared to approximately 19 % sequence variation between *Balamuthia* and *Acanthamoeba* (Booton *et al.*, 2003a). Dissimilarities of OK1 to the other isolates in antimicrobial sensitivity and ability to grow in cell-free medium are probably strain differences. The establishment of additional environmental and clinical isolates in culture will lead to a better understanding of the variation that exists in the species with respect to their ecology, growth requirements and the degree of 16S rRNA gene sequence variability.

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