Isolation of *Balamuthia* Amebas from the Environment[1]

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Investigation into the recent death of a young California child from amebic encephalitis revealed antibodies in the serum and amebas from brain specimens identified as *Balamuthia mandrillaris* [1]. Amebas of this group had been isolated from a mandrill baboon at the San Diego Wildlife Park [6]. At first classified as leptomyxid amebas because of their size and morphology, their pathogenicity, growth at elevated temperature, and live cells as food source were sufficient to designate these as separate organisms; they were given the name of *Balamuthia mandrillaris* in honor of the late Dr. William Balamuth [7]. These amebas have been found in some 90 human cases of encephalitis. The cases have occurred worldwide and antibodies to them can be identified in the serum [4]. Earlier attempts to recover the amebas from environmental samples that may have been implicated in the encephalitis cases had not been successful; nevertheless, a group of 18 soil and water samples from the home and play areas of the California child were collected. From one of them, amebas identified as *Balamuthia* (RP-5) were recovered [3]. A second group of soil samples was collected from a separate location unrelated to any known disease. From these, another ameba (OK-1) was isolated and when compared with the known amebas, it, too, is a *Balamuthia* ameba. The isolation of these two soil amebas and their comparison to the ameba (SAm) from the California child is presented here.

MATERIALS AND METHODS

Ameba isolation. Samples of soil, water and cotton swabs, collected in sterile vials were placed on non-nutrient agar in plates coated with *Escherichia coli* as a food source. Incubation was at 18°C or at room temperature. Multiple transfers of small excised pieces of the agar containing the suspect large amebas located deep within the agar, in regions with relatively few of the other soil organisms, succeeded in freeing the amebas from the ciliates, nematodes, and fungi after some 10–20 passage steps. The bacterial load, however, remained high. To reduce it in preparation for inoculation into cultures of monkey kidney cells (MKC) at 37°C, the amebas were grown on agar plates coated with *Naegleria gruberi* in a thin film of axenic medium (PYG) (Balamuth, W. 1964. Nutritional studies on axenic cultures of *Naegleria gruberi*. *J. Protozool.* **11** (Suppl):19–20). At room temperature, the *N. gruberi* feed on the bacteria, but they do not survive when placed at the higher temperature.

Clonal isolation of the amebas. The large *Balamuthia*-like amebas when grown in cultures of MKC at 37°C completely destroy the cell sheet within 1–2 weeks. Unfortunately, additional small, fast growing amebas, residuals from the soil sample, were also present in the MKC cultures. To separate these two ameba types, the large amebas were selected for cloning by the following procedure. The medium from MKC supporting the growth of the amebas was diluted to a near end-point for the amebas. Drops of 1 µl were placed into each well of a 24-well plate. Each drop was inspected with an inverted microscope. The wells containing a single large trophozoite or one large cyst typical for the *Balamuthia* amebas were seeded with MKC in culture medium and incubated at 37°C. After 7–10 days, in some of the wells, only the large amebas were seen in a single plaque-like area of degenerating MKC. Within a few additional days, the ameba numbers became sufficient for transfer to flasks containing MKC. Although there were no small ameba contaminants in the clinical specimen (SAm), clones were prepared for comparison with those from the soil samples.

Comparisons of the clones. Amebas from clones of the isolated cultures were examined by electron microscopy by the late Dr. Julio Martinez at the University of Pittsburgh School of Medicine. Their growth in the axenic *Balamuthia* medium, BM-3 [5] was determined; their response to antisera from encephalitis victims of *Balamuthia* amebas was observed by indirect immunofluorescent antibody assay; their sensitivity after exposure to a set of antimicrobials was determined; and sequence data was obtained from their extracted DNA.

RESULTS AND DISCUSSION

The initial outgrowth from the soil samples consisted of numerous small amebas, ciliates, nematodes and fungi. Later, large leptomyxid amebas migrated out from the sample location. After weeks, amebas somewhat smaller than the leptomyxids and tubular in shape with multiple projecting pseudopodia were present within and under the agar in the area of the soil sample. With additional time, they moved out into areas of the agar containing relatively few of the other organisms. After multiple passages on agar and successful separation, they were cultivated at 37°C in cultures of MKC. Clones of the amebas were used for the comparative study.

Each of the three amebas, SAm, RP-5, and OK-1, yielded clones from both the trophic and the cystic stages. From the SAm amebas, 7 of 20 trophs and 24 of 45 cysts yielded cultures with an overall yield of 48%. From the RP-5 amebas, 40 of 52 trophs and 3 of 10 cysts gave rise to cultures, a yield of 70%. From the OK-1 amebas, 13 of 35 trophs and 28 of 78 cysts resulted in cultures, a yield of 36%.

The amebas in the cloned cultures readily adapted to growth in the axenic medium BM-3 with the exception that the OK-1 clones were more fastidious. They survived in the BM-3 medium for one or two passages before dying out; they are being maintained on MKC.

Electron microscopic examination of the amebas showed that the fine structure in both the trophozoites and in the cysts of the two environmental isolates was similar to that of the SAm amebas, as well as to other clinical isolates of *Balamuthia* amebas [7].

The response of the SAm, RP-5 and OK-1 ameba clones to indirect immunofluorescent antibody reactions gave positive results against rabbit anti-*Balamuthia* serum and serum from encephalitis victims. Little if any reaction occurred with anti-serum to *Acanthamoeba* or serum from healthy humans.

Ameba growth on MKC cultures in the presence of antimicrobials showed that each was sensitive and failed to grow in the presence of 1 µg/ml of pentamidine isethionate. None of the amebas was sensitive to fluconazole, 5-fluorocytosine, or sulfadiazine at concentrations of 1, 5, or 10 µg/ml in the medium as they continued to feed and proliferate. The antimicrobials amphotericin B and azithromycin, however, yielded variable results in the different experiments with the SAm and RP-5 amebas. The OK-1 clones differed in that the results were variable in the presence of amphotericin and their growth was not inhibited by azithromycin.

Sequence analysis of the DNA showed that the SAm amebas from the California child, the RP-5 amebas from her home, and the OK-1 amebas from an unrelated soil sample are *Balamuthia*. In addition, the sequence data of these three amebas is identical to that of amebas isolated from an encephalitis case that occurred in Nevada [2].

While the amebas isolated from two unrelated soil samples have some differences in their adaptation to growth in BM-3 medium, and

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[1] We dedicate this paper to the memory of Dr. A. Julio Martinez and Dr. Thomas Byers, each a friend and colleague.

to their response to the antimicrobial amphotericin, their overall characteristics and their sequence analysis identify them as *Balamuthia* amebas. These results show that *Balamuthia* amebas are present in soil, and like *Acanthamoeba* and *Naegleria* amebas, they are free-living organisms. The slow growth of the environmental *Balamuthia* and their close association with the other soil organisms makes their isolation a tedious and long-term affair.

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