Mortality of Mice Inoculated with Cryopreserved *Naegleria fowleri*

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Amebae *of Naegleria fowleri,* a free-living opportunistic pathogen, were frozen at -70 °C for 30 months and the viability and infectivity tested at various intervals. The viability of frozen amebae decreased by 22% during the study, with the greatest decrease occurring within the first few months of freezing. The infectivity of the amebae for mice was not reduced by long-term freezing. The results suggest that the more virulent amebae in a population of cells were better able to withstand freezing than the less virulent amebae.

INTRODUCTION

Naegleria fowleri is a free-living, opportunistic ameba that causes a rapidly fatal infection involving the central nervous system, called primary amebic meningoencephalitis. The disease typically occurs in healthy children or young adults with a recent history of swimming in fresh-water, the source of human infection (*1*).

The virulence of *N. fowleri* decreases with prolonged maintenance in axenic culture. However, virulence may be restored by serial passage in mice (*2*). The cryogenic preservation, or freezing, of living cells eliminates the need for continuous maintenance, which invariably leads to genetic variation, as evidenced in *N. fowleri* by the loss of virulence during axenic cultivation. We previously published a procedure for the optimal freezing of opportunistic amebae, including *N. fowleri* (*3*). The purpose of this study was to determine whether the infectivity of *N. fowleri* for mice was reduced by long-term freezing.

MATERIALS and METHODS

Ameba cultivation: The strain of *N. fowleri* used in this study was LEE-M, originally isolated from patient cerebrospinal fluid in Richmond, Virginia, by Clifford Nelson (*4*), and passaged in mice for maximum virulence. Amebae were then maintained in Nelson's medium (*5*; see *6* for composition) for approximately 2 years without passage in mice and were frozen. Amebae were cultivated in 25-cm² polystyrene tissue-culture flasks (Corning Class Works, Corning, New York). Cultures were inoculated with 1×10^5 amebae and incubated at 37 °C.

Ameba freezing: Exponential growth-phase amebae were used for freezing and were rinsed twice with fresh Nelson's medium while in the tissue-culture flasks, and then suspended at 1×10^6 amebae/ml in the freezing medium, which consisted of 12% DMSO, 20% heat-inactivated bovine calf serum, 4% glucose, in Nelson's medium (*3*). Amebae in freezing medium were dispensed (1.0 ml) into cryogenic vials (Corning), placed at -20 °C for 1 h and stored at -70 °C in an ultra-low temperature freezer (Harris Manufacturing Co., North Billercia, Massachusetts). Thawing of the amebae was accomplished by placing the cryovials in a 37 °C waterbath. Viability was determined by exclusion of 0.4% Congo Red prepared in ionized water. The Congo Red solution was mixed with equal volumes of amebae in freezing medium and viability was assessed by light microscopy. Viable amebae appeared pale blue and nonviable cells were reddish brown. Each percent viability given in Table 1 is the average viability of 1,200 amebae (three counts of 200 cells for each of two cryovials).

Mouse inoculation: After thawing, amebae were grown in Nelson's medium for

2 weeks before mouse inoculation. Male, 21-day-old, CD-1 mice were used in all experiments and were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts). They were allowed to adjust to their new environment for 2 or 3 days before experimentation. The mice were given free access to water and feed (Purina Lab Chow, Ralston Purina Corp., St. Louis, Missouri). Mice were inoculated by intranasal instillation of a 10-μl suspension of amebae in Page's ameba saline (*7*). While mice were under anesthesia (Metofane, Pitman-Moore, inc., Washington Crossing, New Jersey), a 10-μl drop containing the desired inoculum was introduced into a single naris using an Eppendorf pipet (Brinkman Instruments, Inc., Westbury, New York). Mice (10 or 15 per group) were inoculated with 1×10^2, 1×10^3, 1×10^4, or 1×10^5 Lee-M strain *N. fowleri.* Deaths were recorded daily and cumulative percent mortality was calculated 28 days, after inoculation. Infection in mice was confirmed by culturing the brain tissue of all dead or dying mice for amebae in Nelson's medium.

RESULTS and DISCUSSION

Table 1 shows that the viability of the frozen amebae decreased by 22% during the 30 months of freezing, with the greatest decrease, 18%, occurring within the first 4 months. Previously, we reported decreases in viability of 19% for *Naegleria australiensis* and 27% each for *N. fowleri* and *Acanthamoeba castellanii* at 12 months of freezing (*3*). The present study confirms that the greatest decrease in viability of frozen opportunistic amebae in these two genera occurs during the first year, and suggests that the first, few months may be critical.

Other investigators have reported 8% viability (*8*) and 63% intact cells (*9*) for *N. fowleri* frozen in liquid nitrogen for 14 days and 7 days, respectively, and an average of 46% viability for nonpathogenic *Naegleria gruberi* frozen in liquid nitrogen for 2 years (*10*). The investigators of these reports described viability in terms of ameba motility (*8,10*) and ameba integrity (*9*). In the present study, we used the exclusion of 0.4% Congo Red together with growth in Nelson's medium to determine viability. We previously evaluated viability staining using 0.4% Trypan Blue and 1% Neutral Red, and found Congo Red to be superior for distinguishing live and dead amebae (*3*).

The results presented in Table 1 show that infectivity of the amebae was not reduced by long-term freezing; rather, it increased during the course of freezing. For example, at a dose of 1×10^3 amebae per mouse, mortality was 33% at 1 month, 50% at 12 months and 90% at 30 months of freezing. It is unlikely that infectivity increased during freezing. What is more likely is that the more virulent amebae in a population of cells were better able to survive freezing than less virulent amebae, making it appear that infectivity increased. The mean time to death also decreased with prolonged freezing, another indication that the more virulent amebae were better able to survive the conditions of freezing and thawing. Previously, it had been reported that *N. fowleri* remained pathogenic to mice after 5 months of storage in liquid nitrogen (*11*). Our study extends further the length of time that *N. fowleri* can be frozen without the loss of infectivity, and at temperatures that are not as low as liquid nitrogen, that is, -196 °C versus -70 °C. A future study could compare the viability and virulence of *N. fowleri* frozen at -70 °C and -196 °C.

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TABLE 1. Mortality of mice inoculated with *Naegleria fowleri* (Lee–M) frozen for 30 months[a]

Months frozen	Percent viability	Cumulative % dead (MTD±SD)[b] Inoculum (amebae/mouse)[c] 1×10^2	1×10^3	1×10^4	1×10^5
1	62±5.4	0	33 (17.4±2.6)	60 (16.7±1.9)	—[d]
4	51±6.9	0	53 (18.8±2.4	87 (14.2±1.9)	—[d]
8	51±9.5	7 (17.0±0)	47 (17.0±3.1)	93 (16.1±2.4)	—[d]
12	47±7.3	13 (25.0±1.0)	50 (22.4±5.1)	93 (12.1±1.6)	—[d]
20	42±8.2	20 (22.0±3.0)	60 (15.9±2.6)	100 (13.2±3.0)	100 (13.6±3.5)
26	49±7.7	20 (14.5±1.5)	70 (14.3±2.0)	90 (16.9±2.4)	100 (11.3±1.5)
30	48±8.9	30 (24.3±2.6)	90 (15.4±2.6)	100 (13.5±1.7)	100 (11.9±1.7)

a The Lee-M strain had been passaged in mice for maximum virulence and then maintained in continuous axenic culture for approximately 2 years prior to freezing. Thawed amebae were grown for 2 weeks prior to inoculation.
b Cumulative % dead calculated 28 days after intranasal inoculation; MTD = mean time to death in days.
c Each experimental group for months 1–12 contained 15 mice, for months 20–30, 10 mice.
d Dose level was not tested.

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