Use of Vero-Cell Cultures to Assess Cytopathogenicity of *Naegleria* Species

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Received: 1993 Jun 08; Revised: 1994 Mar 10

African green-monkey kidney (Vero)-cell cultures were used to evaluate the cytopathogenicity of the known species of *Naegleria*. All six species of *Naegleria,* two pathogenic and four nonpathogenic, produced a cytopathic effect (CPE) in Vero-cell cultures. The time required for amebae to destroy the cell monolayers depended on incubation temperature and ameba:target-cell ratio. This is the first report describing the cytopathic capability of nonpathogenic *N. andersoni* and also the first to demonstrate cytopathogenicity of pathogenic *N. australiensis* and non-pathogenic *N. jadini* and *N. lovaniensis* for Vero-cell cultures.

INTRODUCTION

Naegleria is a genus of free-living ameboflagellates which includes the pathogenic species *N. australiensis* and *N. fowleri* and the nonpathogenic species *N. andersoni, N. gruberi, N. jadini,* and *N. lovaniensis*. *Naegleria fowleri* is responsible for a rapidly fatal human disease known as primary amebic meningoencephalitis [reviewed by John (*1*)]. *Naegleria australiensis* has been isolated from the environment and is pathogenic to mice but has not been associated with human infection.

Various mammalian cell lines have been used to study cell-cell interactions with *Naegleria* and other free-living amebae. However, African green-monkey kidney (Vero) cells have been used by more investigators than any other cell line (*2-6*). Vero-cell monolayers are suitable to assess cytopathogenicity of *Naegleria* species because Vero-cell cultures appear to distinguish degrees of cytopathic effect (CPE) (*5, 6*). Because only *N. fowleri* and *N. gruberi* produce CPE in Vero cultures, the purpose of this study was to evaluate the cytopathic potential of all six species of *Naegleria* for Vero-cell cultures.

MATERIALS and METHODS

Amebae: The species and strains of *Naegleria* used in this study were *N. andersoni* (Aq/2/1D), *N. australiensis (*PP 397), *N. lovaniensis* (Aq/9/1/45D), *N. fowleri* (LEE), *N. jadini* (ATCC 30900), and *N. gruberi* (NEG). Cultures of *N. andersoni, N. australiensis,* and *N. lovaniensis* were kindly supplied by Johan De Jonckheere (Brussels, Belgium), *N. fowleri* by Clifford Nelson (Richmond, VA), and *N. gruberi* by Chandler Fulton (Waltham, MA). *Naegleria jadini* was purchased from the American Type Culture Collection (Rockville, MD). The LEE strain of *N. fowleri* was isolated by Clifford Nelson from the cerebrospinal fluid of a patient who died of primary amebic meningoencephalitis in 1968 at the Medical College of Virginia in Richmond (*7*).

All species of *Naegleria* were grown axenically in Mix ameba medium (*1*), an equal mixture of Balamuth's (*8*) and Nelson's (*9*) media that is 0.55% Panmede liver digest, 0.50% proteose peptone, 0.25% yeast extract, and 0.30% glucose in Page's ameba saline (0.12 g NaC1, 0.004 g $MgSO_4 \bullet 7H_2O$, 0.004 g $CaC1_2 \bullet 2H_2O$, 0.142 g Na_2HPO_4, and 0.136 g KH_2PO_4 per liter of distilled water) (*10*) supplemented with 4% bovine calf serum and 1 μg/m1 hemin. Amebae were cultivated in 25-cm² polystyrene tissue-culture flasks (Corning Glass Works, Corning, NY). Cultures were inoculated with 1×10^5 amebae and incubated at 23, 30 or 37 °C.

Early stationary-growth-phase amebae were harvested by centrifugation (1200 g, 10 min, 20 °C), washed twice in Page's ameba saline, and suspended in the cell culture medium (see below). Cell counts were made with a Coulter counter (Model ZBI; Coulter Electronics, Inc., Hialeah, FL) using settings described elsewhere (*5*).

Vero-cell cultures: Vero-cell stocks were purchased from the American Type Culture Collection and were negative for *Mycoplasma* as demonstrated by the fluorochrome DNA-stain indirect test (Bionique Laboratories, Inc., Saranac, NY) and the adenosine phosphorylase test (MycoTect Kit; GIBCO Labs, Grand Island, NY). Vero cells were cultivated in "growth medium" consisting of Eagle's minimal essential medium (MEM) (using Hanks' balanced salt solution) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (KC Biological, Inc., Lenexa, KS). The growth medium was distributed into 25-cm² Corning tissue-culture flasks. After inoculation with the cells, the cultures were incubated at 37 °C. When the cells formed monolayers, the growth medium was replaced with "maintenance medium" [Eagle's MEM with 0.5% (v/v) FBS]; incubation was at 23, 30, or 37 °C.

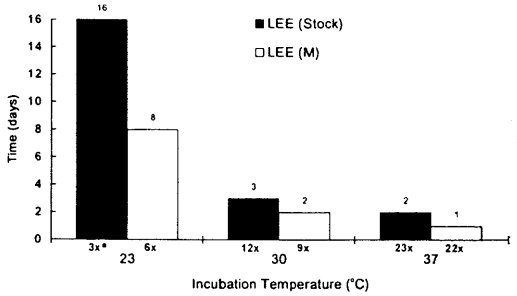

Figure 1. Time required for complete destruction of Vero-cell monolayers by highly and weakly virulent (for mice) *Naegleria fowleri* (LEE strain) at ameba:target cell ratio of 1:10 at different temperatures. There were three Vero-cell cultures per ameba strain per temperature. LEE (Stock), original 1968 isolate, was maintained in Mix ameba medium (see Materials and Methods for composition); LEE (M) was maintained by serial monthly mouse passage to retain maximum virulence. [a]Increase in total number of amebae at time of complete CPE as determined by hemacytometer counts of Vero/*Naegleria* cultures.

Amebae suspended in the cell-culture maintenance medium were added to confluent monolayers at 1:1, 1:10, or 1:100 ameba:Vero-cell ratios and incubated at 23, 30, or 37 °C. The Vero-cell-culture/*Naegleria* system was maintained without antibiotics in an atmosphere of air in tightly capped culture flasks. Cell cultures inoculated with amebae and noninoculated (control) cell cultures were examined daily for CPE by phase-contrast microscopy for as long as 21 days. The levels of CPE produced by *Naegleria* were designated as follows: 0, no CPE (normal cell monolayer); I, slight CPE with few detached cells and few amebae; II, moderate CPE with some detached cells, small plaques, and numerous amebae; III, extensive CPE with many detached cells large plaques, and abundant amebae; IV, only amebae and cellular debris remaining. In this report we present only complete (level IV) CPE. Hemacytometer counts were used to determine the number of amebae present at level IV CPE (Fig. 1).

RESULTS and DISCUSSION

Pathogenicity is the ability of a micro-organism to produce disease whereas cytopathogenicity is the ability to produce pathologic change in cells or a cytopathic effect (CPE) in vitro in cultured cells. Amebae of all six species of *Naegleria* completely destroyed Vero-cell monolayers in 1.0- 10.7 days at an incubation temperature of 30 °C and ameba:target-cell ratios of 1:1, 1:10, and 1:100 (Table 1). The time required for complete CPE depended on the ameba:Vero-cell ratio. The lower the ratio, the less time needed for complete CPE. An ameba:target-cell ratio of 1:100 showed the greatest variation in time for complete CPE, with *N. gruberi* and *N. australiensis* taking 4.0 days and *N. andersoni* 10.7 days. This is the first report of *N. andersoni, N. australiensis, N. jadini,* and *N. lovaniensis* producing CPE in Vero-cell cultures.

Cursons and Brown (*3*) used Vero cultures to assess CPE by *N. gruberi, N. fowleri,* and *N. jadini.* However, they did not obtain CPE with *N. gruberi* and *N. jadini* because the Vero cultures were incubated at 37 °C and the two nonpathogens would not grow at a temperature this high. Erroneously, they concluded that nonpathogenic *Naegleria* would not produce CPE in cell culture and that Vero cultures could be used to distinguish pathogen from nonpathogen. The 37 °C incubation temperature merely indicated temperature tolerance and not pathogenicity. However, by lowering the incubation temperature from 37 to 30 °C, Brown (*11*) showed that nonpathogenic *N. gruberi* would cause CPE in mouse embryo-cell cultures. Not only did *N. gruberi* cause CPE, but it produced CPE sooner than that produced by *N. fowleri* at the same temperature.

By using an incubation temperature of 30 °C and an ameba:Vero-cell ratio of 1:100, the time required for *N. fowleri* to produce complete CPE was 5.7 days (Table 1). Of the six species of *Naegleria,* only *N. andersoni* and *N. jadini* took longer. De Jonckheere (*12*) reported that *N. andersoni* was not cytopathic for Hep G2 cells at 37 °C. We have observed *N. andersoni* to be cytopathic for Vero cells at 37 °C at an ameba:target cell ratio of 1:1, producing complete CPE in 24 h (this study but data not presented), which was the same as for 30 °C. These results provide additional support for the proposal to use Vero-cell cultures to examine cytopathogenicity in *Naegleria* species (*5, 6*).

TABLE 1. Cytopathic effect (CPE[a]) induced by *Naegleria* in Vero-cell cultures incubated at 30 °C.

Naegleria species (strain)	Days[b] required for complete destruction of monolayers		
	Ameba:Vero-cell ratio		
	1:1	1:10	1:100
N. andersoni (Aq/2/1D)	1.0	2.0	10.7±0.5
N. australiensis (PP 397)	1.0	2.0	4.0±0.0
N. fowleri (LEE)	2.0	3.0	5.7±0.5
N. gruberi (NEG)	1.0	2.0	4.0±0.0
N. jadini (ATCC 30900)	3.0	4.0	9.3±0.5
N. lovaniensis (Aq/9/1/45D)	1.0	2.0	5.3±0.5

[a] CPE was assessed using three Vero-cell cultures per species per ameba:target-cell ratio.
[b] Mean number of days±SD; the SD for columns 1:1 and 1:10 is indistinguishable from 0.0.

Naegleria fowleri [LEE (M)], which is highly virulent for mice, was more cytopathic for Vero-cell cultures than was weakly virulent *N. fowleri* [LEE (Stock)] over the range of incubation temperatures tested (23, 30, 37 °C) (Fig. 1). Thus, virulence for mice and cytopathogenicity of a pathogenic species demonstrate a positive correlation. However, pathogenicity for mice and cytopathogenicity for Vero cultures do not correlate completely because the nonpathogenic species, *N. gruberi* and *N. lovaniensis,* required less time than *N. fowleri* to produce complete CPE at 30 °C at all ameba:Vero-cell ratios tested (1:1, 1:10, 1:100) (Table 1).

At lower incubation temperatures, *N. fowleri* multiplies less rapidly, produces fewer amebae, and requires a longer time for complete CPE (Fig. 1). For example, at 23 °C there was a sixfold increase in the total number of LEE (M) amebae by day 8 of incubation when complete CPE occurred. At 30 °C the ninefold increase in amebae caused complete CPE in 2 days and at 37 °C the 22-fold increase produced the same in 1 day. In these experiments amebae were counted with a hemacytometer when complete CPE was recorded. The rate of multiplication most likely accounted for the shorter time needed by the nonpathogenic species of *Naegleria* to produce complete CPE at 30 °C (Table 1). *Naegleria gruberi* and *N. lovaniensis* were able to multiply more rapidly than *N. fowleri* at 30 °C and therefore produced CPE sooner.

We have reported previously (*6*) that the cytopathic ability of *N. gruberi* is enhanced by serial passage in Vero-cell cultures. That is, the longer the amebae were maintained in Vero cultures the more rapidly they produced complete CPE. It would be instructive to know whether the *N. gruberi* amebae with increased cytopathogenicity, resulting from serial cell passage, were pathogenic to mice.

In summary, we have demonstrated that the six known species of *Naegleria,* even the four species nonpathogenic to mice, are able to destroy Vero-cell monolayers, that CPE depends on incubation temperature and ameba:target-cell ratio, that an incubation temperature of 30 °C is better than 37 °C for assessing cytopathogenicity, and that Vero cells are a suitable cell line for studying the cytopathogenicity produced by all species of *Naegleria.* Further cell-culture studies should lead to a better understanding of why both pathogenic and nonpathogenic *Naegleria* are able to destroy cultured mammalian cells but only the two pathogenic species are able to infect mice and produce fatal disease.

ACKNOWLEDGMENTS

We thank Susan Brooks for typing the manuscript and preparing the figure. This investigation was supported by assistance grants R-814327 and R-818106 from the United States Environmental Protection Agency.

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