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# MAMMALIAN CELL CULTURES AFFECTED BY NAEGLERIA GRUBERI

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**Abstract.** Amebae of 8 strains of *Naegleria gruberi* were able to destroy 10 established mammalian cell lines including lung, kidney, ovary, connective tissue, neuroblastoma, and laryngeal and cervical carcinoma cells. The strains of *N. gruberi* varied in their ability to produce a destructive effect (DE) in African green monkey kidney (Vero) cell cultures. However, cell line susceptibility was found to be equivalent when tested with the considerably destructive 1518/1 strain of *N. gruberi*. The Vero cell line proved to be a useful indicator culture for assessing the destructive potential of *N. gruberi* strains. Other factors affecting the extent of DE produced were ameba to mammalian cell ratio and the length of time that amebae were maintained in cell culture.

*Naegleria gruberi* is a ubiquitous free-living ameba—possibly the most common ameba in freshwater (Page 1976). It is also an ameboflagellate and, as such, is able to transform into a transient, nonfeeding, nondividing, biflagellate form (Fulton 1977).

A related species of ameba, *N. fowleri*, produces a fatal human disease known as primary amebic meningoencephalitis (Butt 1966, see John 1982 for review). And until recently it was generally held that only the pathogen could produce a cytopathic effect (CPE) in cultured mammalian cells. In fact, the ability of amebae to produce CPE in cell cultures had been proposed as a way to distinguish pathogen from nonpathogen (Cursons and Brown 1978). However, Brown (1980) was able to show that nonpathogenic *N. gruberi* amebae were capable of producing CPE in mouse embryo cell cultures.

Rather than using cell cultures as an indicator of pathogenicity of free-living amebae, for which they are not, the mammalian cell system should be a useful tool for examining the differences in the cytopathic mechanisms of pathogenic and nonpathogenic *Naegleria*. Therefore, this study was undertaken to evaluate the destructive potential of 8 strains of *N. gruberi* for cultured mammalian cells and to determine the susceptibility of 10 established mammalian cell lines for the destructive capability of *N. gruberi* amebae.

We observed that strains of *N. gruberi* amebae varied in their ability to produce a destructive effect (DE) although different mammalian cell lines exhibited a similar degree of sensitivity to a considerably destructive strain of *N. gruberi*. The DE was also affected by the ratio of amebae to mammalian cells and by the length of time that amebae were maintained in cell culture.

## MATERIALS AND METHODS

**Ameba cultures.** Eight strains of *N. gruberi* were used in this study. Their sources, dates of receipt, and references are given in Table 1.

Amebae were grown axenically, without agitation, in Balamuth medium (Balamuth 1964) in 25 $cm^2$ polystyrene tissue culture flasks (Corning Glass Works, Corning, NY). Cultures were inoculated at a concentration of $1 \times 10^4$ amebae/$cm^2$ and incubated at 30 °C.

Amebae were harvested by centrifugation (1,200 g, 10 min, 20 °C) at early stationary phase growth, washed twice in Page ameba saline (Page 1976), and suspended in Eagle medium. Cell counts were made with a Coulter counter (Model $Z_{BI}$, Coulter Electronics, Inc., Hialeah, FL) using settings reported elsewhere (Weik and John 1977).

**Mammalian cell cultures.** Ten established mammalian cell lines were used in this study. The cell lines and their source are given in Table 2. All cell lines tested negative for the presence of mycoplasmal contamination by either the fluorochrome DNA stain indirect test (Bionique Laboratories, Inc., Saranac, NY) or the adenosine phosphorylase test (MycoTect Kit, GIBCO Labs., Grand Island, NY). The mammalian cells were grown in Eagle minimal essential medium with Hanks balanced salt solution, supplemented with 10% heat-inactivated fetal calf serum (KP Biological, Inc., Lenexa, KS), in 25 cm² Corning tissue culture flasks and incubated at 37 °C. When the cells achieved confluence, the medium was replaced and the fetal calf serum reduced to 0.5—2 %, depending upon the cell line, and incubated at 30 °C.

Amebae, suspended in Eagle medium, were added to confluent monolayers at ameba to mammalian cell ratios of 1 : 1—1 : 100. Mammalian cell cultures containing amebae were maintained without antibiotics in an atmosphere of air in tightly capped tissue culture flasks.

Amebae-inoculated cell cultures and noninoculated control cultures were examined daily for DE by phase-contrast microscopy for 7 days. The degree of DE produced by *N. gruberi* amebae was represented by the following: 0, normal cell monolayer with no DE; I, slight DE with few detached cells and few amebae; II, moderate DE with some detached cells, microscopic plaques, and numerous amebae; III, extensive DE with many detached cells, macroscopic plaques, and abundant amebae; IV, complete breakdown of monolayer so that culture flask contained only amebae and cellular debris.

**Electron microscopy.** For electron microscopy, mammalian cell cultures were grown in 93 mm plastic Leighton tubes containing 5 cm² plastic strips (Costar; Data Packaging Corp., Cambridge, MA). The plastic strips containing cultured mammalian cells and amebae were processed for scanning electron microscopy (SEM).

Briefly, plastic strips were removed from the Leighton tubes, rinsed in phosphate buffered saline, and fixed at 23 °C for 1 h with 2.5 % (v/v) glutaraldehyde in Sorensen phosphate buffer (pH 7.2) containing 1% (w/v) sucrose. After fixation, cultures were rinsed in the phosphate buffer-sucrose solution, dehydrated in ethanol, critical point dried, mounted on stubs, and sputter coated with gold-palladium. Specimens were examined and photographed using a JEOL 35C scanning electron microscope at 15 kV (John et al. 1985).

**Table 1.** Destructive effect of 8 strains of *N. gruberi* for Vero cell cultures[a]

| Strain of *N. gruberi*[b] | Source and date[c] | Reference | No. days for complete destruction of monolayer |
|---|---|---|---|
| S | F. L. Schuster, 1976 | Dunnebacke & Schuster 1974 | 1.2 ± 0.4 |
| 1518/1 | F. L. Schuster, 1976 | Band & Balamuth 1974 | 1.2 ± 0.4 |
| NB-1 | C. Fulton, 1976 | Fulton 1970 | 1.5 ± 0.5 |
| Ng7 | S. L. Chang, 1976 | Chang 1971 | 1.5 ± 0.5 |
| NEG | C. Fulton, 1976 | Fulton 1970 | 1.8 ± 0.4 |
| EGB | F. L. Schuster, 1974 | Schuster & Dunnebacke 1974 | 2.0 ± 0 |
| EGS | F. L. Schuster, 1976 | Schuster & Dunnebacke 1974 | 2.5 ± 0.5 |
| NEG-M | C. Fulton, 1976 | Fulton 1974 | 3.2 ± 0.4 |

[a]DE was assessed using 4 Vero cell cultures per strain of *N. gruberi*.
[b]Amebae were added to Vero cell cultures at a ratio of 1 ameba per target cell and cultures were incubated at 30 °C.
[c]Date and person from whom original stock culture was obtained.

**Table 2.** Susceptibility of 10 mammalian cell lines to the destructive action of *N. gruberi* amebae[a]

| Cell line[b] | No. days for complete destruction of monolayer[c] |
|---|---|
| BHK-21 hamster kidney | 1—2 |
| CHO-K1 hamster ovary | 1—2 |
| HeLa human cervical carcinoma | 1—2 |
| HEp-2 human laryngeal carcinoma | 1—2 |
| L-M (TK⁻) mouse connective tissue | 1—2 |
| L929 mouse connective tissue | 1—2 |
| NB41A3 mouse neuroblastoma | 1—2 |
| Neuro-2a mouse neuroblastoma | 1—2 |
| Vero African green monkey kidney | 1—2 |
| WI-38 human lung | 1—2 |

[a]1518/1 in strain amebae were added to the mammalian cell cultures at a ratio of 1 ameba per target cell and incubated at 30 °C.
[b]Cell lines were purchased from the American Type Culture Collection (Rockville, MD).
[c]DE was assessed using 3 cultures per cell line.



## RESULTS

The destructive effect of *N. gruberi* amebae for Vero cell cultures varied 2 1/2-fold among the 8 strains of amebae examined (Table 1). The number of days required for complete destruction of monolayers ranged from 1.2 to 3.2 with 1.9 days being the mean.

The susceptibility of 10 mammalian cell lines was evaluated using the 1518/1 strain of *N. gruberi*, one of two strains producing the most rapid DE as seen in Table 1. Equivalent susceptibility was observed using this strain of ameba (Table 2). Complete destruction of all monolayers occurred between one and two days after the amebae were added at a ratio of one ameba per target cell.

DE was affected by the ratio of amebae to target cells (Table 3). The greater the number of amebae present, the more rapid the production of DE. For example, with a ratio of one EGB ameba per Vero cell, complete destruction of the monolayer occurred in 2.2 days whereas with one EGB ameba per 10 Vero cells, complete DE did not occur until 5.5 days.

Maintenance of amebae in cell cultures by weekly passage enhanced their ability to destroy Vero cells (Table 4). The longer the amebae were kept in cell culture the more rapidly they produced maximal DE. For example, with an ameba to Vero cell

**Table 3.** Ameba to target cell ratio affecting the destructive action of *N. gruberi* for Vero cell cultures[a]

| Strain of *N. gruberi*[b] | No. days for complete destruction of cells[c] | | |
|---|---|---|---|
| | Ameba to target cell ratio | | |
| | 1 : 1 | 1 : 10 | 1 : 100 |
| 1518/1 | 1.0 ± 0 | 2.5 ± 0.5 | 7 (III) |
| EGB | 2.2 ± 0.4 | 5.5 ± 0.5 | 7 (II) |
| NEG-M | 3.0 ± 0 | 6.2 ± 0.2 | 7 (0) |

[a]DE was assessed using 4 Vero cell cultures per ameba to target cell ratio for each strain of *N. gruberi*.
[b]Amebae were added to Vero cell cultures and incubated at 30 °C.
[c]See Table 4 for an explanation of the degree of DE.

ratio of 1 : 1, complete (IV) DE was produced by day 3 after the first week of passage and by day 1 after the 11th week of passage. Using a ratio of one ameba per 100 Vero cells, DE went from none (0) after the first week of cell passage to moderate (II) in four days after the 12th week of passage.

**Table 4.** Increased destructive ability of *N. gruberi* amebae by serial passage in Vero cell cultures[a]

| Weekly serial cell passage | Extent of DE (day)[b] | | | | | |
|---|---|---|---|---|---|---|
| | Ameba to target cell ratio | | | | | |
| | 1 : 1 | | 1 : 10 | | 1 : 100 | |
| 1 | IV | (3) | IV | (6) | 0 | (7) |
| 2 | IV | (3) | IV | (6) | 0 | (7) |
| 3 | IV | (3) | IV | (5) | I | (7) |
| 4 | IV | (3) | IV | (5) | I | (7) |
| 5 | IV | (2) | IV | (5) | I | (7) |
| 6 | IV | (2) | IV | (5) | I | (6) |
| 7 | IV | (2) | IV | (5) | I | (6) |
| 8 | IV | (2) | IV | (4) | I | (5) |
| 9 | IV | (2) | IV | (4) | II | (5) |
| 10 | IV | (2) | IV | (4) | II | (5) |
| 11 | IV | (1) | IV | (4) | II | (5) |
| 12 | IV | (1) | IV | (3) | II | (4) |
| 13 | IV | (1) | IV | (3) | II | (4) |
| 14 | IV | (1) | IV | (3) | II | (4) |

[a]NEG-M strain amebae were used and DE was assessed with 3 Vero cell cultures per passage, incubated at 30 °C.

[b]0 represents no DE; I represents slight DE with few detached cells and few amebae; II represents moderate DE with some detached cells, microscopic plaques, and numerous amebae; III represents extensive DE with many detached cells, macroscopic plaques, and abundant amebae; IV represents complete breakdown of monolayer

Pl. I shows a clustering of amebae along the margin of a remnant of a Vero cell monolayer where amebae are actively feeding. Numerous amebae are also present in the cell-free area away from the monolayer. Some amebae are present on top of the monolayer remnant. Pl. II shows a single ameba, with multiple extended pseudopodia, on the surface of a Vero cell. The phagocytic amebostomes that we have described for *N. fowleri* amoebae (John et al. 1984, John et al. 1985) are not visible on this amoeba.

## DISCUSSION

Of the five described species of *Naegleria*, namely *N. gruberi*, *N. fowleri*, *N. australiensis*, *N. lovaniensis*, and *N. jadini*, only *N. fowleri* (John 1982) and *N. australiensis* (John and De Jonckheere 1985) are pathogens. Although *N. gruberi* is not pathogenic for mice, clearly it is able to destroy cultured mammalian cells.

Amebae of *N. gruberi* do not grow well, if at all, at 37 °C, the temperature at which most cell cultures are maintained. Because of this, earlier studies reported *N. gruberi* to be noncytopathic for cultured mammalian cells (Cursons and Brown 1978). Amebae of *N. gruberi* were placed in cell cultures and incubated at 37 °C, a temperature at which they could not grow, consequently CPE was not produced.

Brown (1980) was the first to demonstrate the production of CPE by *N. gruberi*



amebae at incubation temperatures of less than 37 °C. As with the others, he also was not able to obtain CPE at 37 °C, but when he lowered the temperature to 30 °C, *N. gruberi* amebae were vigorously cytopathic and destroyed mouse embryo cells more quickly than did *N. fowleri* amebae at the same temperature. We, too, have observed the same. The considerably destructive 1518/1 strain of *N. gruberi* produced complete DE in 10 mammalian cell lines in 1—2 days at 30 °C using a ratio of one ameba per target cell (Table 2). Amebae of *N. fowleri* required 2—3 days to produce complete DE in many of the same cell lines at 37 °C (John 1982). Even more time is required to produce complete DE at a 30 °C incubation temperature.

In addition to the mammalian cell lines that we have tested, other cell lines shown to be susceptible to the cytopathic action of *N. gruberi* amebae are B-103 rat neuroblastoma cells (Marciano-Cabral and Bradley 1982), MRC-5 human lung fibroblasts and RK-13 rabbit kidney cells (Marciano-Cabral et al. 1982), and chick embryo fibroblasts (Zubiaur and Alonso 1983). Obviously, *N. gruberi* is cytopathic for a wide range of cell lines.

Although the 1518/1 strain of *N. gruberi* produced equivalent DE in the 10 mammalian cell lines we examined, the different strains of *N. gruberi* varied in their ability to produce DE in Vero cell cultures (Table 1). The length of time required for complete DE to occur ranged from 1.2 to 3.2 days with 1.9 days being the mean.

All of our *N. gruberi* strains produced DE in Vero cell cultures. Brown (1980), however, reported having two strains of *Naegleria* which did not produce CPE in mouse embryo cells. Hence, we suggest that Vero cell monolayers are a suitable cell line in which to examine cytopathogenicity of *N. gruberi* amebae. Vero cells appear to be a discriminating cell line because degrees of the destructive effect were detected as well.

The mechanism(s) by which *N. gruberi* amebae produce DE is (are) not well understood. However, ameba-target cell contact is required before a destructive effect can occur. The destruction of cultured cells by *N. gruberi* amebae has been attributed to phagocytosis alone (Brown 1980) and to amebamediated cell lysis following contact (Marciano-Cabral and Bradley 1982, Marciano-Cabral and Fulford 1986). In contrast, *N. fowleri* amebae destroy cultured cells by trogocytosis (Brown 1979), a process by which portions of the target cell are engulfed by well-organized structures known as amebostomes (John et al. 1985, Marciano-Cabral and John 1983). In the present study, amebostomes were not seen on the amebae of *N. gruberi* (Pl. II), and cell lysis was not evidenced because amebae were seen to attack remnants of monolayers along their intact margins (Pl. I).

*Naegleria* amebae are also reported to contain a cytopathogenic material (NACM), which has been characterized as an infectious agent which, when purified from amebal lysates, produces cytopathology in cell culture monolayers (Dunnebacke and Schuster 1985). NACM has been described from both pathogenic and nonpathogenic *Naegleria* species but, so far, only from *Naegleria* amebae.

The ameba to target cell ratio affects the length of time required for amebae to produce DE. This would be expected if DE were the result of cell—cell contact and to a lesser extent if DE were the result of cytotoxins being released by amebae into the medium. There is no evidence that *N. gruberi* amebae produce cytolytic or cytotoxic substances into the extracellular environment.

The destructive ability of *N. gruberi* amebae was increased by serial passage in Vero cell cultures. For example, with a ratio of one ameba per 10 Vero cells, amebae produced complete DE in 6 days after one week in cell culture and in 3 days after 12 weeks in Vero cell culture.

It would be instructive to know if *N. gruberi* amebae with increased destructive

ability, resulting from serial cell passage, are pathogenic for mice. There is one report in which *N. gruberi* amebae with enhanced cytopathogenicity were inoculated intranasally into young mice with no deaths resulting (Zubiaur and Alonso 1983). However, the cell cultures had been incubated at 30 °C and, therefore, the amebae would not have been able to survive the higher 37 °C body temperature of mice. The experiment that remains to be done is one in which a strain of *N. gruberi* is adapted to grow at 37 °C, maintained in mammalian cell culture to enhance cytopathogenicity, and then inoculated intranasally into mice. Would these "nonpathogenic" amebae produce primary amebic meningoencephalitis similar to that caused by *N. fowleri*?

In summary, we have surveyed a number of *N. gruberi* strains and mammalian cell lines for destructive ability and susceptibility and have found that the degree of DE produced depends upon the strain of ameba and cell line used, ameba to target cell ratio, and the length of time that amebae were maintained in cell culture.

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## КУЛЬТУРЫ КЛЕТОК МЛЕКОПИТАЮЩИХ ПОРАЖЕННЫЕ *NAEGLERIA GRUBERI*

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**Резюме.** Амебы 8 штаммов *Naegleria gruberi* способны разрушать 10 линий клеток млекопитающих включая клетки легких, почек, яичников, соединительной ткани, нейробластомы и карциномы. Штаммы *N. gruberi* отличались по способности к деструкции (DE) в культуре клеток почек африканской обезъяны (Vero). Чувствительность клеточных линий оказалась одинаковой при использовании деструктивного штамма 1518/1 *N. gruberi*. Клеточная линия Vero может быть использована как индикатор деструктивных способностей штаммов *N. gruberi*. Помимо этого на уровень DE влияют отношение количества амеб и клеток линий и продолжительность совместного инкубирования.

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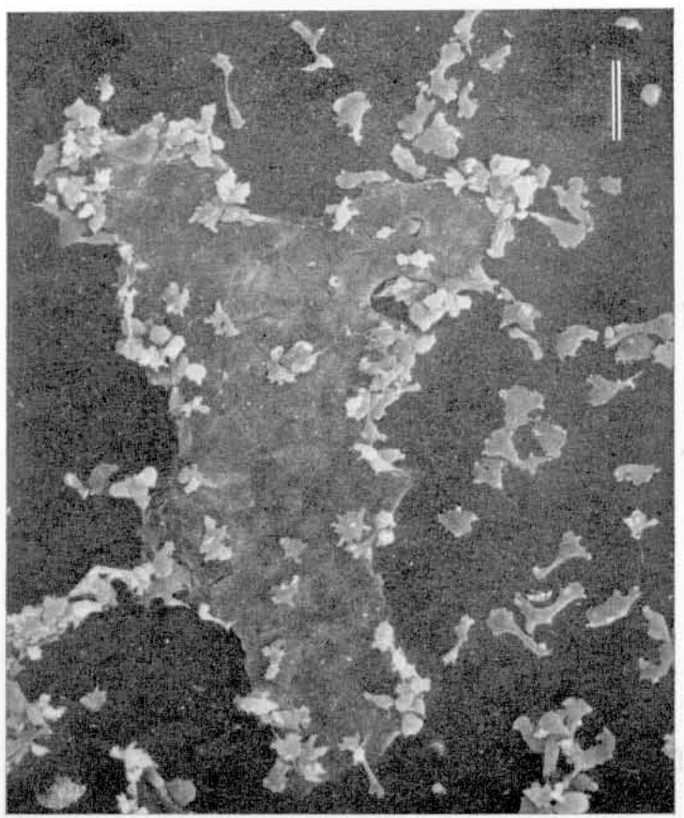
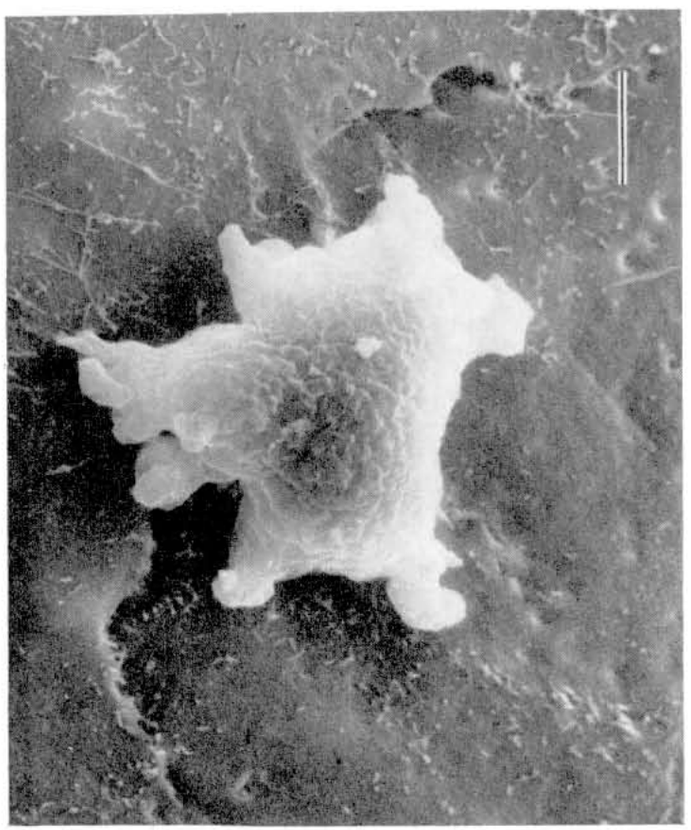


SEM of remnant of Vero cell monolayer with *N. gruberi* (EGB strain) amebae clustered along the margin. Bar. = 5 μm.





SEM of an *N. guberi* (S strain) ameba, with multiple extended pseudopodia, on the surface of a Vero cell. The ameba lacks the phagocytic amebostomes described for *N. fowleri*. Bar = 5 μm.