APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1986, p. 1133–1137
0099-2240/86/051133-05$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 51, No. 5

Cytopathology of Pathogenic and Nonpathogenic *Naegleria* Species for Cultured Rat Neuroblastoma Cells

FRANCINE M. MARCIANO-CABRAL* AND DAVID E. FULFORD

Department of Microbiology and Immunology, Virginia Commonwealth University/Medical College of Virginia, Richmond, Virginia 23298

Received 7 August 1985/Accepted 25 February 1986

The cytopathology for rat neuroblastoma cells (B-103) and the pathogenicity for B6C3F1 mice of four species of *Naegleria* have been compared. Both live amoebae and cell-free extracts of *N. australiensis*, *N. fowleri*, *N. gruberi*, and *N. lovaniensis* added to ^{51}Cr-labeled B-103 cells caused release of radiolabel. All four species of *Naegleria* exhibited surface extensions termed food cups. Only *N. fowleri* and *N. australiensis* were pathogenic for mice. Electron microscopic observations of cultures of either *N. australiensis* or *N. lovaniensis* with B-103 cells established that the cytopathology involved lysis of the B-103 target cells.

The factors associated with pathogenicity of *Naegleria fowleri*, the etiologic agent of primary amoebic meningoencephalitis, are not well understood. Characteristics of *N. fowleri* such as ability to grow above 37°C, growth phase of the amoebae, and means of evasion of the immune system have been proposed to be determinants of pathogenesis (14, 18). Other factors such as differences in cytolytic enzyme activity (3, 5, 22) and increased levels of sphingomyelinase activity (13) have been suggested as possible virulence factors. In the present investigation we have examined a number of properties of four species of *Naegleria* in an attempt to gain insight into the determinants responsible for disease production; these include cytopathogenicity in vitro of live amoebae and amoebic lysate and the presence of food cups on the surface of amoebae. The four species of *Naegleria* investigated were *N. fowleri*, *N. australiensis*, *N. lovaniensis*, and *N. gruberi*.

N. fowleri is thermophilic, pathogenic for humans and experimental animals, and cytopathic for cultured mammalian cells (2, 18, 21). *N. australiensis*, originally isolated from flood waters in Australia, reportedly exhibits low to moderate pathogenicity for mice, is antigenically and biochemically distinct from *N. fowleri* (6–8), and is cytopathic for cultured cells. *N. lovaniensis*, another environmental isolate, is thermotolerant and somewhat related to *N. fowleri* antigenically, but is not pathogenic for mice (20). Finally, *N. gruberi* is mesophilic, is not pathogenic for mice, but is cytopathic for cultured mammalian cells (16).

The LEE strain of *N. fowleri* (ATCC 30894) was initially grown at 37°C axenically in Nelson medium in 75-cm^2 plastic culture flasks (Falcon Plastics, Oxnard, Calif.) as previously described (9). *N. australiensis* PP397 (ATCC 30958) (6), *N. lovaniensis* (Aq/9/1/45D) (20), and *N. gruberi* EGB (19) were initially grown in Balamuth medium supplemented with 2% calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 0.2% hemin (Sigma Chemical Co., St. Louis, Mo.) in 75-cm^2 plastic culture flasks (Falcon Plastics). *N. australiensis* and *N. lovaniensis* were grown at 37°C. *N. gruberi* was grown at 30°C. Cytopathogenicity (18) and pathogenicity studies were performed using *Naegleria* grown in an equal mixture of Nelson medium and Balamuth medium (4). Transmission (18) and scanning (17) electron microscopy of amoebae cultures was conducted on amoebae grown in the Nelson-Balamuth medium.

TABLE 1. Cytopathic effect of live *Naegleria* amoebae or cell extracts for ^{51}Cr-labeled B-103 cells

Amoeba	% Specific release of ^{51}Cr[a] ± SE			
	6 h/extract[b]	6 h/cells[c]	12 h/cells[c]	18 h/cells[c]
N. fowleri LEE	18.8 ± 0.6	15.0 ± 5.7	31.6 ± 10.3	34.9 ± 6.5
N. australiensis PP397	12.2 ± 1.9	30.2 ± 12.9	35.0 ± 10.2	43.3 ± 10.1
N. lovaniensis Aq/9/1/45D	10.9 ± 1.0	34.8 ± 5.3	46.8 ± 2.2	53.2 ± 5.6
N. gruberi EGB	16.1 ± 1.6	15.3 ± 3.2	18.3 ± 2.5	21.2 ± 6.5

[a] Percent specific release of ^{51}Cr = [counts per minute of supernatant/(counts per minute of supernatant + counts per minute of pellet)] × 100 − spontaneous release. Spontaneous release (median) = 1,951 cpm; maximum release (median) = 33,250 cpm.
[b] Cell extracts were prepared by three cycles of freeze-thawing, sonication, and centrifugation at 1,000 × *g* for 20 min (11). Data are presented as percent specific release of ^{51}Cr per milligram of protein.
[c] Amoebae to target cell ratio (1:1), 2 × 10^5 amoebae to 2 × 10^5 B-103 cells. Values are percent specific release of ^{51}Cr from labeled B-103 cells (11).

All four species of *Naegleria* tested were cytopathic for B-103 rat neuroblastoma cells as demonstrated by the specific release of ^{51}Cr from the labeled neuroblastoma cells. All species demonstrated a maximum cytopathic activity by 18 h. *N. fowleri*, *N. australiensis*, and *N. lovaniensis* induced similar release of chromium at 37°C. *N. gruberi* induced less release of ^{51}Cr at 37°C than the other species (Table 1). Cell extracts of the four species of *Naegleria* were cytopathic for the chromium-labeled B-103 neuroblastoma cells. Lysates of *N. fowleri* and *N. gruberi* contained more cytotoxic activity than the lysates of *N. australiensis* and *N. lovaniensis*, based on specific activity.

We have previously shown that cytopathogenicity in vitro by live amoebae and amoebic extracts of several strains of

* Corresponding author.

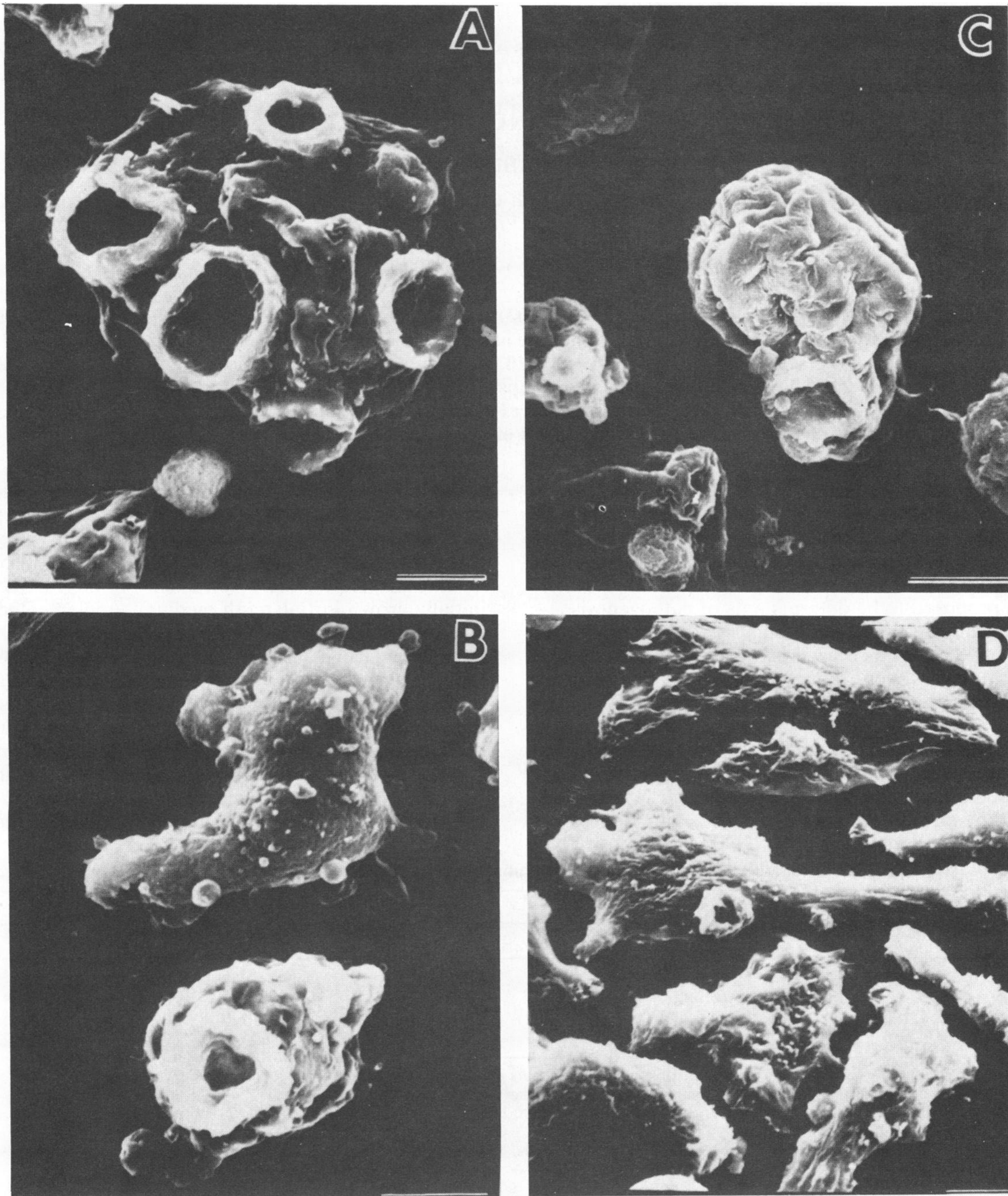

FIG. 1. Scanning electron microscopy of *Naegleria* amoebae. Trophozoites of pathogenic and nonpathogenic species expressing surface stomas or food cups. (A) *N. fowleri* LEE; (B) *N. australiensis* PP 397; (C) *N. gruberi* EGB; (D) *N. lovaniensis* Aq/9/1/45D. Bar, 5 μm.

N. fowleri does not correlate with pathogenicity in mice (11, 18). A similar finding was noted in this study when live amoebae of thermophilic environmental isolates and a nonpathogenic species were compared. Moreover, the relative cytopathic effect in vitro of amoebic extracts of all four *Naegleria* species did not correlate with pathogenicity in vivo. All species, including the nonpathogenic *N. lovaniensis* and *N. gruberi*, produced a profound cytopathic effect in vitro. The somewhat lower level of ^{51}Cr release induced by live *N. gruberi* may be due to the culture conditions of these amoebae. *N. gruberi* are normally grown at 30°C and as a result may be not as active at 37°C, the assay temperature, as the other species which are grown at 37°C (16, 18).

Intranasal instillation of 2×10^6 *N. fowleri* LEE per mouse produced 75% mortality with a mean time to death of 17 days. As few as 5,000 *N. fowleri* amoebae per mouse inoculated intracranially produced 100% death in B6C3F1 mice in 8 days. *N. australiensis* did not produce death in B6C3F1 mice when inoculated intranasally. One of eight mice died after intracranial inoculation of 5×10^3 *N. australiensis*. Amoebae were not recovered from any of this

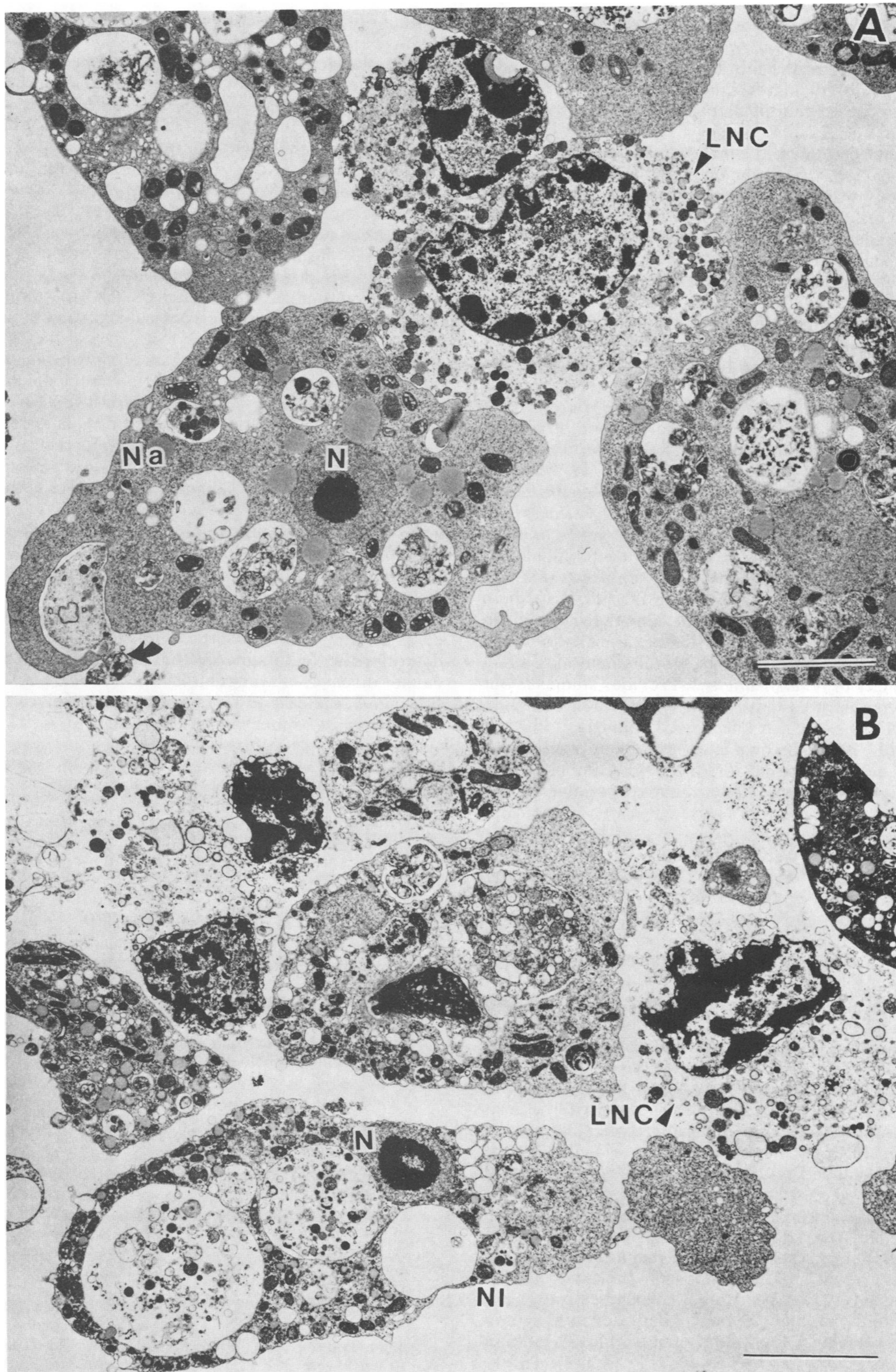

FIG. 2. (A) Lysis and phagocytosis (arrow) of target cell debris by *N. australiensis* (Na) amoebae after 6 h at 37°C. (B) Lysis of B-103 target cells by *N. lovaniensis* (Nl) amoebae at 37°C. LNC, Lysed nerve cell; N, nucleus. Bar, 5 μm.

group of animals. Two of eight mice died after intracranial inoculation of 2×10^6 *N. australiensis* in 9 days. Amoebae were recovered from the brains of both animals. The thermophilic environmental isolate, *N. lovaniensis*, and the mesophilic environmental isolate, *N. gruberi*, were not pathogenic for B6C3F1 mice.

In the present study, *N. australiensis* PP397 was weakly pathogenic for mice. One of the eight animals died, and one of eight animals was sick but recovered during the 21-day observation period after intracranial inoculation of 5,000 amoebae. In a second group of mice inoculated intracranially with 2×10^6 amoebae, two of eight animals died. Previous workers have reported that *N. australiensis* is weakly to moderately pathogenic for mice (6–8). There are several factors which may contribute to differences in mice mortality studies: (i) mouse strain, (ii) mouse age, (iii) sex, (iv) growth phase of the amoebae at the time of inoculation, (v) the amoebae growth medium, and (vi) axenic versus monoaxenic cultivation of the amoebae. Previous workers have used weanling male NMRI mice. Both age and strain are known to be important variables in susceptibility to *Naegleria* infections (14). DeJonckheere et al. (8) reported that intranasal inoculation of mice with *N. australiensis* PP397 grown in monoaxenic culture with *Escherichia coli* resulted in four deaths of five mice, whereas inoculation of the same amoebae grown in the absence of bacteria failed to kill mice. These investigators also reported that *N. australiensis* PP397 axenically grown in SCGYEM medium produced 60% mortality, whereas amoebae grown in YPNFS medium did not cause deaths after intracranial inoculation of amoebae (8). The growth medium may affect the pathogenicity of *N. australiensis*. Previous studies in our laboratory demonstrated that *N. fowleri* grown in Balamuth medium or Nelson medium killed mice challenged intranasally and that *N. gruberi* grown in either Balamuth or Nelson medium was not pathogenic for mice (4). Composition of growth medium was not a determinative variable in our studies because all four strains were cultivated in the same medium.

Cultures of amoebae were examined by scanning electron microscopy to determine whether cytoplasmic extensions, termed food cups, were present on the surface of trophozoites. Food cups (one to five in number) were detected on axenically cultured *N. fowleri* LEE (Fig. 1A). *N. australiensis* also exhibited food cups (one to three in number) (Fig. 1B). An occasional *N. gruberi* (Fig. 1C) and *N. lovaniensis* (Fig. 1D) amoeba exhibited a single food cup when axenically cultured in the combination Nelson-Balamuth medium. Endocytic stomas or food cups have been noted on the surface of *Entamoeba histolytica* (12) and *N. fowleri* (15, 17). Gonzalez-Robles and Martinez-Palomo suggested that different types of stomas appear to be associated with internalization of liquid and particulate components (12). In the present study, both *N. fowleri* and *N. australiensis* displayed several food cups on the surface of the amoebae. Food cups were rarely observed on *N. gruberi* or *N. lovaniensis*. Stomas which were smaller in size and fewer in number were occasionally observed on *N. gruberi* and *N. lovaniensis*. There is not a definitive correlation between the presence of food cups on the amoebae and pathogenicity in vivo. Other studies in our laboratory also indicate that more pathogenic, mouse-passaged strains of *N. fowleri* exhibited fewer food cups than axenically cultured amoebae (15).

Amoebae can be readily distinguished from neuroblastoma cells by their distinct protozoan nucleus, upon examination of thin sections with an electron microscope (Fig. 2). The amoebae of *N. australiensis* and *N. lovaniensis* became attached to the neuroblastoma cells within 1 h at 37°C. The plasma membrane and cytoplasm of a target cell were damaged in the proximity of the amoebae. The endoplasmic reticulum of the nerve cells was dilated and round. The target cells in contact with amoebae were lysed as early as 6 h after cocultivation. The prominent feature of the cytopathic action of *N. australiensis* and *N. lovaniensis* on neuroblastoma cells was lysis of the target nerve cell (Fig. 2). The nucleus of the target cell remained intact. The amoebae appeared to ingest the target cell debris by phagocytosis or to draw in lysed target cell material.

N. fowleri, unlike *N. australiensis*, destroys target cells by trogocytosis, a process of piecemeal ingestion of target cells by food cups (1, 17). This may suggest that the cytoplasmic extensions termed food cups on *N. australiensis* and *N. lovaniensis* may play a role in attachment of amoebae to substrates in addition to being used for ingestion of particulate material. Alternatively, cytolytic substances may be released at the attachment site between food cups and target cells. Target cells were lysed by *N. australiensis* and *N. lovaniensis* within 6 h. This mechanism of target cell injury more closely resembles that caused by *N. gruberi*, a nonpathogenic amoeba which lyses target cells on contact (16), than that caused by *N. fowleri* (18). Release of ^{51}Cr from labeled nerve cells confirmed early damage of target cells by *N. australiensis* and *N. lovaniensis*. The ability to kill cells in vitro, growth at 37°C, and the presence of food cups on the amoeba surface do not appear to be the determinative factors involved in the ability of *Naegleria* to cause disease. Pathogenicity in *Naegleria*, as in *E. histolytica*, appears to be complex and involves more than one substance or mechanism (10).

This research was supported by Public Health Service research grant 1R01AI21055 from the National Institute of Allergy and Infectious Diseases, Biomedical Research Grant 2S07RR5724 from the National Institutes of Health, and training grant CA09210 from the National Cancer Institute.

We thank David T. John for providing cultures of *N. australiensis* and *N. lovaniensis*.

LITERATURE CITED

1. **Brown, T.** 1979. Observations by immunofluorescence microscopy and electron microscopy on the cytopathogenicity of *Naegleria fowleri* in mouse embryo-cell cultures. J. Med. Microbiol. **12**:363–371.
2. **Carter, R. F.** 1970. Description of a *Naegleria* sp. isolated from two cases of primary amoebic meningoencephalitis and of the experimental pathological changes induced by it. J. Pathol. **100**:217–244.
3. **Chang, S. L.** 1974. Etiological, pathological, epidemiological and diagnostical considerations of primary amoebic meningoencephalitis. Crit. Rev. Microbiol. **3**:135–159.
4. **Cline, M. L., F. Marciano-Cabral, and S. G. Bradley.** 1983. Comparison of *Naegleria fowleri* and *Naegleria gruberi* cultivated in the same nutrient medium. J. Protozool. **30**:387–391.
5. **Cursons, R. T. M., T. J. Brown, and E. A. Keys.** 1978. Virulence of pathogenic free-living amoebae. J. Parasitol. **64**:744–745.
6. **DeJonckheere, J. F.** 1981. *Naegleria australiensis* sp. nov., another pathogenic *Naegleria* from water. Protistologica **17**:423–429.
7. **DeJonckheere, J. F., M. Aerts, and A. J. Martinez.** 1983. *Naegleria australiensis*: experimental meningoencephalitis in mice. Trans. R. Soc. Trop. Med. Hyg. **77**:712–716.
8. **DeJonckheere, J. F., P. Pernin, M. Scaglia, and R. Michel.** 1984. A comparative study of 14 strains of *Naegleria australiensis* demonstrates the existence of a highly virulent subspecies: *N.*

australiensis italica n. spp. J. Protozool. **31**:324–331.
9. **Duma, R. J., W. I. Rosenblum, R. F. McGhee, M. M. Jones, and E. C. Nelson.** 1971. Primary amoebic meningoencephalitis caused by *Naegleria*. Ann. Intern. Med. **74**:923–931.
10. **Feingold, C., R. Bracha, A. Wexler, and D. Mirelman.** 1985. Isolation, purification and partial characterization of an enterotoxin from extracts of *Entamoeba histolytica* trophozoites. Infect. Immun. **48**:211–218.
11. **Fulford, D. E., S. G. Bradley, and F. Marciano-Cabral.** 1985. Cytopathogenicity of *Naegleria fowleri* for cultured rat neuroblastoma cells. J. Protozool. **32**:176–180.
12. **Gonzalez-Robles, A., and A. Martinez-Palomo.** 1983. Scanning electron microscopy of attached trophozoites of pathogenic *Entamoeba histolytica*. J. Protozool. **30**:692–700.
13. **Hysmith, R. M., and R. C. Franson.** 1982. Elevated levels of cellular and extracellular phospholipases from pathogenic *Naegleria fowleri*. Biochim. Biophys. Acta **711**:26–32.
14. **John, D. T.** 1982. Primary amebic meningoencephalitis and the biology of *Naegleria fowleri*. Annu. Rev. Microbiol. **36**: 101–123.
15. **John, D. T., T. B. Cole, and F. M. Marciano-Cabral.** 1984. Sucker-like structures on the pathogenic amoeba *Naegleria fowleri*. Appl. Environ. Microbiol. **47**:12–14.
16. **Marciano-Cabral, F., and S. G. Bradley.** 1982. Cytopathogenicity of *Naegleria gruberi* for rat neuroblastoma cell cultures. Infect. Immun. **35**:1139–1141.
17. **Marciano-Cabral, F., and D. T. John.** 1983. Cytopathogenicity of *Naegleria fowleri* for rat neuroblastoma cell cultures: scanning electron microscopy study. Infect. Immun. **40**:1214–1217.
18. **Marciano-Cabral, F., M. Patterson, D. T. John, and S. G. Bradley.** 1982. Cytopathogenicity of *Naegleria fowleri* and *Naegleria gruberi* for established mammalian cell cultures. J. Parasitol. **68**:1110–1116.
19. **Schuster, F. L.** 1969. Intranuclear virus-like bodies in the ameboflagellate *Naegleria gruberi*. J. Protozool. **16**:724–727.
20. **Stevens, A. R., J. F. DeJonckheere, and E. Willaert.** 1980. *Naegleria lovaniensis* new species: isolation and identification of six thermophillic strains of new species found in association with *Naegleria fowleri*. Int. J. Parasitol. **10**:51–64.
21. **Visvesvara, G. S., and C. S. Callaway.** 1974. Light and electron microscopic observations on the pathogenesis of *Naegleria fowleri* in mouse brain and tissue culture. J. Protozool. **21**:239–250.
22. **Wong, M.M., S. L. Karr, and C. K. Chow.** 1977. Changes in the virulence of *Naegleria fowleri* maintained *in vitro*. J. Parasitol. **63**:872–878.