PRIMARY AMEBIC MENINGOENCEPHALITIS AND THE BIOLOGY OF NAEGLERIA FOWLERI

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INTRODUCTION

Primary amebic meningoencephalitis (PAM) is a rapidly fatal human disease caused by the ameboflagellate Naegleria fowleri. The disease first was detected in man 17 years ago by Fowler & Carter in Australia (56). A year later in 1966 three fatal infections were described from Florida by Butt (10).
The symptomatology of these cases was remarkably similar to that observed in Australia. Although it was not apparent then, the seven cases in Australia and Florida provided almost a complete array of the important clinical and pathological features of the disease.

Notable, also, was the indication that infection was acquired by intranasal instillation during swimming. Butt (10) recognized the discovery of a new disease in Australia and Florida by contributing a new name, primary amebic meningoencephalitis.

*Naegleria fowleri* was named in honor of Dr. Malcom Fowler who first recognized the disease it caused (13). This has been the only species of *Naegleria* to be isolated from victims of PAM. Synonyms for *N. fowleri* are *N. aerobia* (114) and *N. invades* (24). Nonpathogenic species of *Naegleria* include *N. gruberi* (see 58), possibly the most common ameba in fresh water (101), *N. thornoni* (113), and *N. lovaniiensis* (118). *N. jadini*, isolated from swimming pool water in Belgium, is only slightly pathogenic for mice (140).

*Naegleria* is an ameboflagellate; it belongs to a family of amebae (Vahlkampfiidae) whose members can transform from amebae into flagellates (101). The *Naegleria* flagellate is a transient, nonfeeding, nondividing form. The life cycle of *Naegleria* also includes cyst formation and excystment by amebae (Figure 1). *Naegleria* flagellates do not encyst; only the amebae divide and are able to encyst.

**HUMAN INFECTION**

Amebic infections of the central nervous system may be caused by the parasitic ameba *Entamoeba histolytica* or by the opportunistic free-living amebae *Naegleria fowleri* or *Acanthamoeba* spp. *E. histolytica* may produce a brain abscess after extraintestinal invasion and subsequent hematogenous
spread. *Acanthamoeba* spp. produce an illness known as granulomatous amebic encephalitis (91). Disease usually occurs in chronically ill or debilitated individuals, some of whom may be undergoing immunosuppressive therapy. Invasion of the central nervous system appears to be hematogenous, arising from a primary lesion of the skin, lungs, or kidneys. In this review, discussion is limited to *N. fowleri* and the disease it produces.

**Clinical Features**

PAM typically occurs in healthy children or young adults with a recent history of swimming in freshwater lakes or pools. The disease is rapidly fatal, usually producing death within 72 hr after the onset of symptoms. Infection follows inhalation of water containing amebae or flagellates. It also has been suggested that inhaling cysts, during dust storms, for example, could lead to infection (84, 108).

Amebas penetrate the nasal mucosa and the cribiform plate and travel along the olfactory nerves to the brain. Amebas first invade the olfactory bulbs and then spread to the more posterior regions of the brain. Within the brain they provoke inflammation and cause extensive damage to the tissue (13).

The clinical course is dramatic. Symptoms begin with severe frontal headache, fever (39–40°C), and anorexia. This is followed by nausea, vomiting, and signs of meningeal irritation. Involvement of the olfactory lobes may cause disturbances in the sense of smell or taste and may be noted early in the course of the disease. Visual disturbances may occur. The patient may experience confusion, irritability, and restlessness and may become irrational before lapsing into coma. Generalized seizures also may be present. In order of frequency of occurrence, the more important symptoms include headache, anorexia, nausea, vomiting, fever, and stiff neck (49, 93).

**Pathology**

The gross pathologic findings in PAM are remarkably constant. The cerebral hemispheres usually are edematous and swollen. Meninges are diffusely hyperemic with a slight purulent exudate. The cortex contains many focal superficial hemorrhages. The olfactory bulbs exhibit marked involvement with hemorrhage, necrosis, and purulent exudate (14, 49, 93).

Microscope examination reveals many amebae in the subarachnoid and perivascular spaces. Presumably, the perivascular spaces provide a path of migration for the amebae, and the blood vessels supply the oxygen needed by these aerobic organisms. In fewer numbers, amebae are found clustered within the brain tissue and in the purulent exudate of the meninges and brain substance. Within the exudate some amebae may be seen engulfed by macrophages. Many amebae are observed to contain phagocytosed cellular
debris and erythrocytes. The purulent exudate contains numerous polymorphonuclear and mononuclear leukocytes (14, 93).

The cortical gray matter is a preferred site for amebae development; consequently, severe involvement occurs in the cerebral hemispheres, cerebellum, brain stem, and upper portions of the spinal cord. Encephalitis ranges from slight amebic invasion and inflammation to massive invasion with purulent, hemorrhagic necrosis. Typically, the olfactory bulbs exhibit extensive amebic invasion, hemorrhage, and an inflammatory exudate; the involvement here is greater than in other areas of the brain. Infection of the central nervous system with *N. fowleri* may be described best as an acute, hemorrhagic, necrotizing meningoencephalitis (14, 93).

Focal demyelination in the white matter of the brain and spinal cord has been described (27, 51). Curiously, demyelination occurred in the absence of amebae or cellular infiltration. Chang (27) suggests that demyelination is caused by a phospholipolytic enzyme or enzyme-like substance produced by actively growing amebae present in the adjacent gray matter.

Myocarditis has been described in some patients dying of PAM (14, 89). It has been suggested (89) that the observed myocarditis may be caused by a circulating myotoxin either produced by amebae in the brain or released by rapidly degenerating invading amebae. However, evidence has not yet been produced to substantiate either hypothesis.

**Diagnosis**

The diagnosis of PAM is made by microscope identification of living or stained amebae in patient cerebrospinal fluid. Motile amebae are readily seen in simple wet-mount preparations of spinal fluid. Amebae can be distinguished from other cells by their limax (L. sluglike) shape and progressive movement. It is not necessary to warm the slide since amebae remain fully active at room temperature. Refrigeration of the spinal fluid is not recommended as this may kill the amebae (13, 93).

Spinal fluid smears may be stained with Wright or Giemsa stains (108). The bacterial Gram stain is of little positive value since heat fixing causes the amebae to stain poorly and to appear as degenerate cells. Giemsa- or Wright-stained amebae have considerable amounts of sky-blue cytoplasm and relatively small, delicate, pink nuclei. Mononuclear leukocytes, on the other hand, have large purplish nuclei with only small amounts of sky-blue cytoplasm. Acridine orange has also been used to distinguish *N. fowleri* amebae from leukocytes (97). Using acridine orange and ultraviolet microscopy, amebae stain brick-red with pale green nuclei in contrast to leukocytes, which are bright green.

Amebae also may be cultured by placing some of the spinal fluid on non-nutrient agar (1.5%) spread with a lawn of *Enterobacter aerogenes* or
Escherichia coli and incubated at 37°C. The amebae will grow on the moist agar surface and will utilize the bacteria as a source of food.

Clinically, PAM very closely resembles fulminating bacterial meningitis, and the laboratory findings are also similar. The cerebrospinal fluid is purulent or sanguinopurulent with leukocyte counts, predominantly neutrophils, ranging from a few hundred to over 20,000 cells/mm³. Spinal fluid glucose levels are low, and generally, protein content is increased. Typically, Gram-stained smears and cultures of spinal fluid are negative for bacteria (14, 93).

**Treatment**

At present, no satisfactory treatment for PAM exists. The antibiotics used to treat bacterial meningitis are ineffective in naeglerial infection, as are the antiamebic drugs. Amphotericin B, a drug of considerable toxicity, is an antinaeglerial agent for which there is some evidence of clinical effectiveness. Both known survivors of PAM were treated with amphotericin B, given intravenously and intrathecally (2, 15); the only patient in the United States known to have survived naeglerial infection (15) also was given parenteral miconazole and oral rifampin. The in vitro testing of a highly virulent human isolate of *N. fowleri* demonstrated that amebae were extremely susceptible to amphotericin B [minimal inhibitory concentration (MIC), 0.15 μg/ml], somewhat susceptible to miconazole (MIC, 25 μg/ml), and resistant to rifampin (MIC, ≤100 μg/ml) (119). Mice were protected by treatment with amphotericin (7.5 mg/kg/day) but not by treatment with lower doses of amphotericin B alone or in combination with miconazole (100 mg/kg) or rifampin (220 mg/kg). It appears, then, that amphotericin B currently is the most effective treatment for PAM. Amphotericin B is administered intravenously at a dose of 1 mg per kg of body weight daily, with intrathecal inoculations of 0.1 mg on alternate days (14).

Amphotericin B is a polyene compound that acts upon the plasma membrane, disrupting its selective permeability, and causing leakage of cellular components (82). When exposed to amphotericin B, amebae round up and do not form pseudopodia. Membrane-related changes, evident by electron microscopy, include enhanced nuclear plasticity, increased amount of smooth and rough endoplasmic reticulum, decreased food vacuole formation, and production of blebs of the plasma membrane (110).

Lipopolysaccharide has been shown to afford mice some protection for several days after challenge with *N. fowleri* (1). Slightly better protection was provided by treatment of mice with Δ⁹-tetrahydrocannabinol (105).

Tetracycline (127) and rifampin (126) have been shown to act synergistically with amphotericin B to protect mice against *N. fowleri* infection. In the tetracycline study, chemotherapy was started 72 hr after the mice had
been infected intranasally. Survival was 38% for amphotericin B-treated mice and 88% for mice treated with the amphotericin B-tetracycline combination (127). Combination therapy is a reasonable approach to treatment for patients with PAM.

EPIDEMIOLOGY AND ECOLOGY

Geographic Distribution

Although it is a relatively rare disease, PAM has been reported worldwide. Most of the reports have been from developed rather than from developing nations, perhaps because of a greater awareness of the disease in these countries and not because of greater incidence.

Cases have been reported from Belgium, Czechoslovakia, Great Britain, Northern Ireland, India, Australia, New Zealand, New Guinea, Uganda, Nigeria, Venezuela, Panama, Puerto Rico, and the United States. As of October 1981, 108 cases of *Naegleria* infection have been described worldwide; 49 of these have been from the United States. The states reporting cases have been New York, Virginia, North Carolina, South Carolina, Georgia, Florida, Mississippi, Arkansas, Texas, Arizona, Nevada, and California.

The majority of patients with naeglerial infection have had a history of recent swimming in fresh water during hot summer weather. In Richmond, Virginia, infection in 14 of the 16 cases probably was acquired in two man-made lakes located within a few miles of each other (12, 51, 108).

Over a 3-year period, 16 young people died in Czechoslovakia after swimming in the same heated, chlorinated, indoor swimming pool (23). Similar fatal cases have been reported following swimming in swimming pools in Belgium (68), England (11), and New Zealand (34); in hot springs in California (15) and New Zealand (31); in lakes in Florida (10, 16), Texas (16), Arkansas (143), and South Carolina (39); and in streams in Belgium (129), New Zealand (32), and Mississippi (87).

Infection has not always been acquired through swimming, though. The South Australia and northern Nigeria cases have occurred in rather arid regions where swimming is not often indulged. The proposed means of infection for these areas has been face washing and bath-related activity (2, 85) and inhalation of dust-borne cysts (84).

Environmental Isolations

*Naegleria fowleri* has been isolated from a variety of environmental sources. During the summer of 1971, Nelson (98) isolated *N. fowleri* from a water sample taken from a pond where a victim of PAM swam in 1967. This constituted the first isolation of *N. fowleri* from an environmental source. Today, the pond no longer is used for swimming but has since been con-
verted into an ornamental pool in a suburban housing development. However, another man-made lake in the immediate neighborhood still attracts swimmers daily throughout the summer months despite the fact that five children have died of PAM after swimming there; the last case occurred 14 years ago in 1968 (12, 51, 108).

Additional environmental isolations of *N. fowleri* have been made from tap water in South Australia (69); heated indoor swimming pools in Czechoslovakia (79) and England (11); lakes in Florida (139), Nigeria (86), and Poland (80); warm effluents from power plants in Florida (121), Texas (121), and France (47) and from factories in Belgium (41); sewage and sludge samples in India (115), Korea (117), and Nigeria (86); soil samples in South Australia (3), New Zealand (34), and Nigeria (86); and river water in Spain (95).

*N. fowleri* clearly is cosmopolitan in its environmental distribution. Curiously, though, most of the isolations have been made from habitats manipulated or altered in some way by man or, and this appears to be an important factor, from habitats subjected to warming, whether man-made or natural.

On two occasions, *N. fowleri* has been isolated from the nasal passages of young children. Amebae pathogenic for mice were isolated by nasal swab from a 7-year-old boy in Richmond, Virginia (28, 112), and from two young children, under 10 years of age, in Zaria, Nigeria (84). Although the Nigerian children had symptoms of upper respiratory tract infection, none of the children had signs or symptoms of meningitis, and the Richmond child had no evidence of infection of any sort.

**Animal Infection**

There are only a few reports, apart from those describing human infections, in which *Naegleria* has been isolated from animals in nature. They include the recovery of *Naegleria* from the gills of fish (122), from snails (81, 103), and from various reptiles (57). *Acanthamoeba*, a related freshwater ameba, has been detected in visceral lesions of a dog (4), in pulmonary lesions of a buffalo (53), in a bull (96), in renal granulomas in a gold fish (131), and cultured from clinical specimens and tissues of a variety of diseased domestic animals (77).

There is no evidence for the existence of an animal reservoir or carrier host for *N. fowleri*. Nonetheless, until adequate surveys have been carried out, the possibility cannot be excluded. Animals that need to be examined include aquatic insects (and insect larvae), crustaceans and mollusks, fishes, amphibians and aquatic reptiles, waterfowl and shore birds, and aquatic or diving mammals such as beavers, otters, and muskrats. All the above animals could encounter *N. fowleri* naturally and, particularly the migratory species, could serve as transport hosts. Perhaps this accounts for the observation made by DeJonckheere & Van De Voorde (45) that *N. fowleri* could
be isolated from waters where old factories discharged thermal waste, but not from the discharges of newer factories. The animal(s) involved may require a period of adjustment before investigating a newly built facility.

Researchers have used several laboratory animal species as models for their investigations of experimental PAM. *Naegleria fowleri* produces fatal meningoencephalitis in mice (13, 18, 29, 92, and others), guinea pigs (18, 30, 104), rabbits (29), monkeys (29, 141), and sheep (144). The recent significant finding that sheep are susceptible to *N. fowleri* following intranasal instillation of amebae suggests the possible occurrence of PAM among domestic livestock.

Chick embryos also are susceptible to *N. fowleri* infection (66). Ameba inoculated on the chorioallantoic membrane disseminate to the brain, liver, spleen, and lungs. Inoculation with as few as 10 amebae will cause death of the chick embryo within 5 days.

The mouse is uniquely appropriate as an experimental model for studying PAM. The basic features of the disease in man and mouse are the same with respect to portal of entry, incubation period, and invasion of the nasal mucosa, cribiform plate, and olfactory bulbs with subsequent spread to more distant areas of the brain (92).

Fatal naeglerial meningoencephalitis in mice is route and dose dependent. Most of the animal studies have employed either intranasal or intracerebral routes of inoculation, which require fewer amebae to establish infection than do the other routes. Clinical symptoms and death were produced in mice inoculated intravenously and intraperitoneally with large doses of amebae (1, 13). A dose of $5 \times 10^6$ amebae per mouse was administered subcutaneously without deaths occurring (1). However, since the outcome of an infection with *N. fowleri* is dose dependent, it is reasonable to propose that a larger dose (or a more virulent strain) could produce the disease in mice via subcutaneous inoculation.

It is also possible to produce infection in mice after intranasal instillation with the flagellate stage of *N. fowleri* (116). However, since flagellates are rather unstable, they undoubtedly reverted to the ameba stage in the nasal passages, and the amebae subsequently invaded the nasal mucosa. Flagellates were not seen in brain smears of mice dying from meningoencephalitis; only amebae were noted (116).

Cysts of *N. fowleri* never have been observed in human brain tissue or in tissues from experimentally produced animal infections. This is in direct contrast to infection with *Acanthamoeba* in which cysts are routinely detected.

**Control and Prevention**

Because of the swimming-related nature of naeglerial infection, many swimming areas have been subjected to intense investigation. And although it is
true that \textit{N. fowleri} has been isolated from some swimming areas (described above), there are several reports in which environmental sampling of swimming pools has failed to produce \textit{N. fowleri} (19, 42, 48, 70, 88). Obviously, some factors favor the development of \textit{N. fowleri} in swimming areas. These include warm temperature, presence of an adequate food source (organic matter or bacteria), insufficient residual free chlorine, minimal competition from other protozoans, and probably optimal pH and oxygen levels.

With the present limited understanding of the ecology of \textit{N. fowleri}, practical measures for prevention and control of naeglerial infection include education of the public, awareness within the medical community, and adequate chlorination (10 ppm) of public swimming facilities (44). Chang (26) has demonstrated that \textit{N. fowleri} amebae are sensitive to drying, high temperature (>51°C), low temperature (<10°C), and especially freezing. Cysts are sensitive to desiccation (nonviable in <5 min), survive poorly at 0°C, but tolerate high temperatures (51–65°C). Cysts of \textit{N. fowleri} have been stored for up to 8 months with excysting amebae retaining virulence (132). Effectiveness of chlorination is dependent upon residual free chlorine, temperature, and pH (26).

\textit{Legionella pneumophila}, the agent of Legionnaires' disease, has been shown to be pathogenic for \textit{N. gruberi} and \textit{N. jadini} (107). It has been suggested that \textit{Naegleria} (and \textit{Acanthamoeba}) possibly may be natural hosts for \textit{L. pneumophila}, and that human infection may be acquired not by inhaling free legionellae but by inhaling amebae full of legionellae (50–1000 or more bacteria per ameba) (107). Our own studies (D. T. John, N. C. Mobley, unpublished data) indicate that \textit{N. fowleri} is more susceptible to infection by \textit{L. pneumophila} than is \textit{N. gruberi}. Perhaps \textit{Legionella} (and/or other bacteria or viruses) serves as a natural biological control of \textit{N. fowleri} and accounts for the relative scarcity of \textit{N. fowleri} in the environment.

Considering the millions of persons who swim each summer in streams, lakes, and swimming pools, the probability of becoming infected is rather remote. For example, despite the extensive distribution of \textit{N. fowleri} in Florida's freshwater lakes (121, 139), estimates are that the risk of acquiring naeglerial infection is about one case per 2.6 million exposures (138).

**PHYSIOLOGY/METABOLISM**

**Nutrition and Growth**

Perhaps the simplest way to grow \textit{N. fowleri} is on the surface of non-nutrient agar (1.5%) spread with living or dead \textit{Enterobacter aerogenes} or \textit{Escherichia coli}. Under these conditions, the amebae feed upon the bacteria, and as growth enters stationary phase and the food supply is used up, they
begin to encyst. Cysts, if kept from drying out, will remain viable for months, possibly years.

Unfortunately, the presence of bacteria too often hampers a variety of quantitative studies. Therefore, several liquid media have been developed for the axenic cultivation of *N. fowleri* (5, 17, 26, 99). In general, these media contain a phosphate buffer and either liver extract, yeast extract, peptone, or casein derivative bases supplemented with serum.

Liquid axenic media developed by Balamuth (5), Červa (17), Chang (26), and Nelson & Jones (99) have been used for the cultivation of *N. fowleri* (20, 40, 52, 133). Growth of *N. fowleri* under agitated and unagitated culture conditions was compared using these four axenic media (63). The less enriched media of Červa (17) and Nelson & Jones (99) supported greater cell yields under both agitated and unagitated culture conditions.

The liver infusion-proteose peptone-yeast extract-glucose-calf serum medium of Balamuth (5) originally was developed for the axenic cultivation of *N. gruberi*; hence, it is not surprising that it does not support good growth of *N. fowleri*. The two species are distinct organisms with different nutritional requirements. In contrast to *N. gruberi*, *N. fowleri* grows best in less enriched media (63, 110). Červa medium contains only casitone and horse serum (17) and Nelson medium has liver digest, glucose, and calf serum (133).

By using agitated cultures and Nelson medium, it is possible to obtain large quantities (3 x 10⁹ amebae/liter) of *N. fowleri* (133). At 37°C, the mean generation time is 5.5 hr for exponentially growing cells. There is only slight utilization of glucose, and amino acids appear to serve as carbon and energy sources.

Physical factors shown to affect the growth of *N. fowleri* in liquid axenic cultures include pH, temperature, viscosity, and dissolved inorganic salts. The pH optimum for growth initiation in agitated cultures is 5.5 (133), and 6.5 in unagitated cultures (21, 133). The pH of Nelson culture medium increases about 2 U during 96 hr of growth (133). However, if the medium is adjusted to maintain a constant pH, there is no change in growth of *N. fowleri*, indicating that the pH increase of the culture medium does not limit ameba growth (83).

Optimal temperature for the growth of *N. fowleri* is dependent upon the composition and concentration of the culture medium, viability of the amebae in the inoculum, and size of the inoculum (20). It has been suggested that a threshold inoculum is necessary for growth initiation of *N. fowleri* in broth cultures (40). The suggestion was made to account for inconsistent ameba growth in cultures inoculated with less than 10⁴ ameba/ml. However, the results of a study in which flasks are inoculated with 10²–10⁶ ameba/ml suggest that a threshold inoculum level does not exist (83). Rather, there was a maximum population density or carrying capacity.
for the medium. Cultures inoculated with $10^2$ grew about 10 generations before entering stationary growth phase, whereas cultures inoculated with $10^6$ amebae/ml grew only one generation.

A 0.5% concentration of methylcellulose in liquid medium does not inhibit the growth of *N. fowleri* (21). By comparison, *N. gruberi* growth is inhibited by concentrations of methylcellulose greater than 0.2%.

Serum appears to be an important component of the liquid media used for axenic cultivation of *N. fowleri*. Various kinds and concentrations of serum have been used. Several forms of calf serum and sera from other vertebrate species have been evaluated for their ability to support growth of *N. fowleri* in Nelson medium (64, 73). Of the 17 sera tested, calf serum supported the greatest cell yields ($1.48 \times 10^6$ amebae/ml), whereas fetal calf serum produced the lowest cell yields ($2.09 \times 10^5$ amebae/ml). Between these two and ranked in order of decreasing cell yield are pig, dialyzed calf, monkey, newborn calf, lamb, turtle, dog, chicken, mouse, rabbit, frog, horse, gamma globulin-free calf, fish, and human sera.

Hemin (1 μg/ml) has been shown to replace serum as a growth requirement for *N. gruberi* in axenic liquid cultures (6). However, the same is not true for cultures of *N. fowleri*, although hemin, in addition to the serum, will enhance growth (75). A semi-defined medium without serum and containing hemin has been described (37). A curious observation about the Richmond, Virginia, cases is that 14 of the 16 infections were acquired in two man-made lakes, which occur in the vicinity of the first iron smelter to be built in the United States. The water in some of the small streams in the area often appears reddish because of its iron content. Perhaps the presence of iron or other heavy metals in nature provides an environment favorable to the growth of *N. fowleri*.

**Cell Differentiation**

*Naegleria fowleri* is an ameboflagellate and, therefore, it is able to transform from ameba to flagellate and revert to ameba, and to encyst and under favorable conditions to excyst (Figure 1). Yet, apart from testing to verify the identity of *N. fowleri* isolates, there are no definitive physiological studies of differentiation in *N. fowleri*. Cell differentiation in *N. gruberi* has been reviewed by Fulton (58).

Trophozoites of *N. fowleri* are long and slender (8–15 μm) and have progressive flowing movement. The resting nucleus is spherical with a sharply defined nuclear division is promitotic, in which the nucleolus elongates and divides into two polar masses and the nuclear membrane remains intact (101).

Cysts are spherical, often clumped closely together, and 7–10 μm in diameter (13). Ultrastructure examination reveals an average of less than
2 mucoid-plugged pores per cyst and a relatively thin cyst wall, a feature that makes *N. fowleri* cysts susceptible to desiccation (109).

When trophozoites are suspended in distilled water or non-nutrient buffer (58) they transform into temporary flagellated forms. The flagellate has an elongate, pear-shaped body, usually two flagella of equal length, a nucleus in the narrower anterior region, and no cytostome (13, 101). The ultrastructure of *N. fowleri* flagellates is that of a typical eucaryotic protist (102). There is a distinct nuclear membrane and prominent nucleolus, numerous vacuoles and cytoplasmic inclusions, pleomorphic mitochondria, and some rough endoplasmic reticulum. Amebae of *N. fowleri* (LEE strain) became flagellates 150 to 180 min after transfer to non-nutrient buffer. Basal bodies, a rootlet, and flagella are formed quickly after an initial lag of 90 min.

Carter (13) observed that transformation occurred after 20 hr in distilled water, and that the flagellates persisted until 48 hr. Transformation was unreliable below 21°C and was better, with 50% flagellates, at 37°C.

We have noted (D. T. John, N. C. Mobley, unpublished data) that several of our axenically cultured *N. fowleri* isolates no longer differentiate into flagellates when amebae are placed in non-nutrient buffer (Table 1). Appar-

<table>
<thead>
<tr>
<th>Strain of ameba</th>
<th>Isolation</th>
<th>Flagellates (%)</th>
<th>Maximum transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lovell</td>
<td>Florida</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>KUL</td>
<td>Belgium</td>
<td>129</td>
<td>4</td>
</tr>
<tr>
<td>LEE(M-11)c</td>
<td>Virginia</td>
<td>51</td>
<td>4</td>
</tr>
<tr>
<td>LEE(ATCC-30894)</td>
<td>Virginia</td>
<td>51</td>
<td>4</td>
</tr>
<tr>
<td>TY</td>
<td>Virginia</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>6088</td>
<td>California</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>WM</td>
<td>Virginia</td>
<td>51</td>
<td>4</td>
</tr>
<tr>
<td>HB-5</td>
<td>Texas</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>CJ</td>
<td>Virginia</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>0 359</td>
<td>Belgium</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>GJ</td>
<td>Florida</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>HB-4</td>
<td>North Carolina</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>HB-4(M-8)c</td>
<td>North Carolina</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>NF66</td>
<td>Australia</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>NF69</td>
<td>Australia</td>
<td>13</td>
<td>0</td>
</tr>
</tbody>
</table>

aAmebae were grown at 37°C in Nelson medium with 2% calf serum. At 72 hr, medium was removed, amebae were rinsed three times with Page saline, covered with cold (4°C) Page saline, and vortexed. Amebae (~10⁶ cells/ml) were placed in 125-ml Erlenmeyer flasks and shaken at 37°C in a gyrotory shaker (New Brunswick) at 100 rpm. Flasks were examined hourly for 8 hr by placing samples in D’Antoni’s iodine and counting the percentage of flagellates. Cultures not producing flagellates were held and examined periodically to 36 hr.

bTime in hours after the initiation of differentiation (time zero).

cM-11 and M-8 denote amebae that had been serially mouse-passaged 11 and 8 times, respectively.
ently, some axenic populations of *N. gruberi* amebae fail to transform (100) and must be maintained with bacteria to do so. Fulton (58), however, states that axenically grown *N. gruberi* differentiate to flagellates but do so more slowly than bacteria-grown cells. Whatever the reason, it would be instructive to examine transformation in our non-differentiating *N. fowleri* isolates after growth with bacteria.

**Macromolecular Composition**

Changes in cell composition of *N. fowleri* are related to culture age (135). For agitated axenic cultures, average cell dry mass remained constant during log growth at 150 pg/ameba, but decreased 30% during stationary growth at 96 hr. During log growth 80–85% of the cell dry mass was protein (120 pg/ameba). Cell dry mass and protein of *N. fowleri* are about 70% of values reported for *N. gruberi* (134).

During log and stationary growth phases, carbohydrate content averaged 15 pg/ameba, and RNA was about 18 pg/ameba (135). RNA content for *N. gruberi* was about 8 pg/ameba (134). Perhaps the over twofold greater RNA value for the smaller pathogenic *N. fowleri* reflects different biosynthetic capabilities by maintenance of a larger ribosome complement.

Total DNA content was 0.2 pg/ameba during log growth; it doubled during transition from log phase to stationary phase and then gradually decreased to nearly initial levels. The peak in DNA content corresponded to an increase in the average number of nuclei per ameba; nuclear number then decreased as cells entered stationary growth phase (135).

**Cell Membrane and Agglutination**

Concanavalin A (Con A) agglutinates *N. gruberi* but does not agglutinate *N. fowleri* (76, 120). Agglutination is time and temperature dependent and Con A concentration and ameba concentration dependent over certain ranges. At least $10^6$ amebae/ml are needed for maximum agglutination, and Con A concentrations higher than 100 µg/ml do not appreciably increase agglutination. These results indicate that only *N. gruberi* has appreciable quantities of N-acetyl glucosamine and mannose-like residues manifested on the cell surface. *Naegleria lovaniensis*, which is nonpathogenic for mice, is also agglutinated by Con A (118).

**Respiratory Metabolism**

As an opportunistic parasite, *N. fowleri* lives in an oxygen-rich environment (the brain) and so one would expect it to have an aerobic metabolism. The synonym *N. aerobia* (114) recognized the aerobic nature of the organism, in contrast to the anaerobic nature of strictly parasitic amebae. Unlike *Entamoeba histolytica*, an anaerobic parasitic ameba that lacks mito-
chondria (59), *N. fowleri* lives in aerobic aqueous environments and has many mitochondria (90, 111).

Whole cell respiration rates were measured polarographically throughout the growth cycle of *N. fowleri* (136). Under agitated culture conditions, amebae consumed 30 ng of O/min/mg of cell protein during log growth. Under similar conditions *N. gruberi* amebae consumed 80 ng of O/min/mg of cell protein (137). The lower oxygen consumption, and most likely oxygen requirement, by *N. fowleri* probably explains the presence of the pathogen in heated waters where dissolved oxygen concentrations are substantially reduced.

Respiratory rate gradually declined during stationary growth phase. The reduction in respiratory rate may involve respiratory control since further increases in respiratory rate did not occur in spite of fresh oxygen supplies (136).

The respiratory process of isolated *N. fowleri* mitochondria is similar to classical mammalian cell mitochondria. Oxidation was "coupled" to phosphorylation (ATP formation) as shown by the two- to threefold increase in respiration upon addition of phosphate acceptor or uncoupling agent. Difference spectra of oxidized and dithionite-reduced mitochondria showed distinct absorption bands of flavins, c-type, b-type, and a-type cytochromes (136).

**VIRULENCE AND IMMUNITY**

*Mechanisms of Pathogenesis*

The determinants of virulence, invasiveness, and pathogenicity of *Naegleria fowleri* are largely unknown. Electron microscope studies of experimentally induced PAM in mice have demonstrated phagocytic activity by amebae (90, 130).

*N. fowleri* causes a destructive cytopathic effect in cultured mammalian cells, and it is generally supposed that this cytopathic activity is associated with trophozoite pathogenicity. Opinion, though, is divided on the mechanism of cell damage. Chang (25) reported that supernatant medium from *N. fowleri*-infected cultures of mammalian cells induced cell degeneration when fresh cultures were inoculated with it, which suggests that amebae secrete cytolytic or cytotoxic enzyme-like substances. He concluded (27) that the pathogenicity of cytopathic effect of *N. fowleri* can be attributed to a phospholipolytic enzyme released by the amebae during active growth.

Wong et al (142) observed differences in cytolytic enzyme synthesis between highly virulent and low-virulent strains of *N. fowleri*; for example, highly virulent strains produced a magnitude more catalase than low-virulent strains. Cursons et al (33) found that *N. fowleri* produced a greater
amount of phospholipase A than did nonpathogenic *N. gruberi*. Hysmith et al (67) have measured sphingomyelinase levels in *Naegleria* culture media and determined that sphingomyelinase activity was approximately 100-fold greater in culture medium of virulent *N. fowleri* than it was in culture media of low-virulent *N. fowleri* or *N. gruberi*. Increased levels of sphingomyelinase activity in culture medium may be directly associated with the demyelination in brain and spinal cord that has been described in PAM (27, 51).

Brown (7, 8) suggests that the cytopathogenicity of *N. fowleri* in secondary mouse-embryo cells does not involve ameba-associated cytotoxic activity, but depends on normal phagocytic function. Amebae that were immobilized and agglutinated by specific antiserum exhibited no cytopathic activity, although they remained alive and were in constant contact with the host cells. Cytochalasin B, shown to inhibit phagocytosis in amebae, inhibited the cytopathogenicity of *N. fowleri* when it was added to cell culture medium. Brown concludes that *N. fowleri* amebae attack and destroy cultured mouse-embryo cells by a phagocytosis-like mechanism alone, without the aid of ameba-associated cytotoxic or cytolytic agents.

We have examined the susceptibility of various mammalian cell lines to the cytopathic activity of a highly virulent strain (HB-4) of *N. fowleri*. With a multiplicity of infection of 1, complete destruction of the monolayers occurred from 24 hr (BHK-21, Vero, WI-38 cells) to 72 hr (L929, Ktk-, Neuro-2a cells) after inoculation of amebae for cultures incubated at 37°C (Table 2). Cytopathic effect occurs to a lesser extent and later for cultures inoculated with a low-virulent strain of *N. fowleri*.

Brown (9) also has described the cytopathogenicity of nonpathogenic *N. gruberi* in several mammalian cell lines, cultured at 30 rather than 37°C. Again, cytopathic effect was caused by phagocytosis, and he suggests that temperature sensitivity has been a significant factor in the reported differences in cytopathogenicity between *N. fowleri* and *N. gruberi* amebae.

*Naegleria*-induced cytopathic effect has also been attributed to the transmission of infectious cytopathogenic material from ameba to susceptible avian and mammalian cultured cells (52). The infectious material, present in both *N. fowleri* and *N. gruberi*, appears to be a protein with an estimated molecular weight of 50,000. It is capable of sustaining itself in cell culture and in serial passage through multiple dilutions.

Several factors have been shown to affect the virulence of *N. fowleri* for mice; they include incubation temperature, growth phase, and strain of ameba (61). Amebae cultured at 30 and 37°C were more virulent than ameba growth at 23 and 44°C. Mortality was greater for mice inoculated with amebae harvested at late logarithmic and early stationary growth
Table 2 *Naegleria fowleri* (HB-4 strain)-induced cytopathic effect (CPE) in cultured mammalian cells.a

<table>
<thead>
<tr>
<th>Cell line b</th>
<th>Animal species and tissue</th>
<th>Time (hr) for maximum CPE c</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>Baby golden hamster kidney IV</td>
<td></td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney IV</td>
<td></td>
</tr>
<tr>
<td>WI-38</td>
<td>Human lung IV</td>
<td></td>
</tr>
<tr>
<td>CF-3</td>
<td>Human foreskin IV</td>
<td></td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster ovary IV</td>
<td></td>
</tr>
<tr>
<td>HeLa S3</td>
<td>Human epithelioid carcinoma, cervix IV</td>
<td></td>
</tr>
<tr>
<td>HEp-2</td>
<td>Human epidermoid carcinoma, larynx IV</td>
<td></td>
</tr>
<tr>
<td>Mv 1 Lu</td>
<td>Mink lung IV</td>
<td></td>
</tr>
<tr>
<td>NB41A3</td>
<td>Mouse neuroblastoma IV</td>
<td></td>
</tr>
<tr>
<td>L929</td>
<td>Mouse connective tissue IV</td>
<td></td>
</tr>
<tr>
<td>Ltk-</td>
<td>Mouse neuroblastoma IV</td>
<td></td>
</tr>
</tbody>
</table>

a HB-4 is a highly virulent strain of *N. fowleri* isolated by R. B. Finley in July 1977 from patient spinal fluid, before death, at Winston-Salem, NC.

b Cells were grown and maintained in MEM with fetal calf serum. All cell lines were obtained from the American Type Culture Collection (Rockville, MD) except CF-3 (Noble Fdn., Ardmore, OK) and Ltk- (S. Kit, Baylor, Houston, TX), a thymidine kinase deficient L929 derived cell line.

c Multiplicity of infection was 1; four 25-cm² tissue culture flasks per cell line; 37°C incubation temperature; CPE of IV represents complete breakdown of monolayer so that culture flask contains only amebae and cellular debris.

phases than it was for amebae harvested at early logarithmic and late stationary growth phases.

The incubation of *N. fowleri* amebae with mouse anti-*N. fowleri* serum has been shown to have a rather dramatic effect upon ameba virulence and subsequent mouse mortality after intravenous inoculation (62). The mortality rate was 15% for mice inoculated with immune serum-treated amebae compared with 95% mortality rate for mice inoculated with amebae incubated in normal (nonimmune) mouse serum. Viability testing, using trypan blue exclusion, immediately after incubation of amebae with serum, showed that the immune mouse serum caused greater damage to the amebae, with ~5% nonviable cells as compared to less than 1% nonviable amebae after incubation with normal mouse serum.

Virulence varies greatly among the different human isolates of *N. fowleri*. Table 3 gives the mortality for mice inoculated intranasally with 5 X 10³ amebae/mouse of 13 different strains (isolates) of *N. fowleri*. Cumulative percent mortality at 28 days ranged from 0–100% and mean time to death
NAEGLERIA MENINGOENCEPHALITIS

Table 3  Mortality for mice inoculated intranasally with $5 \times 10^3$ amebae/mouse of 13 strains of *Naegleria fowleri*<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain of ameba</th>
<th>Initial isolation</th>
<th>Date of isolation</th>
<th>Ref.</th>
<th>Cumulative percent dead&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean time to death (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJ</td>
<td>Virginia</td>
<td>1967</td>
<td>51</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>KUL</td>
<td>Belgium</td>
<td>1973</td>
<td>129</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>TY</td>
<td>Virginia</td>
<td>1969</td>
<td>51</td>
<td>10</td>
<td>14.0</td>
</tr>
<tr>
<td>0 359</td>
<td>Belgium</td>
<td>1970</td>
<td>68</td>
<td>15</td>
<td>13.3</td>
</tr>
<tr>
<td>LEE(ATCC-30894)</td>
<td>Virginia</td>
<td>1968</td>
<td>51</td>
<td>20</td>
<td>17.8</td>
</tr>
<tr>
<td>NF66</td>
<td>Australia</td>
<td>1966</td>
<td>13</td>
<td>75</td>
<td>16.8</td>
</tr>
<tr>
<td>Lovell</td>
<td>Florida</td>
<td>1974</td>
<td>40</td>
<td>85</td>
<td>15.5</td>
</tr>
<tr>
<td>HB-4</td>
<td>North Carolina</td>
<td>1977</td>
<td>None</td>
<td>85</td>
<td>7.2</td>
</tr>
<tr>
<td>WM</td>
<td>Virginia</td>
<td>1969</td>
<td>51</td>
<td>90</td>
<td>11.1</td>
</tr>
<tr>
<td>NF69</td>
<td>Australia</td>
<td>1969</td>
<td>13</td>
<td>90</td>
<td>10.6</td>
</tr>
<tr>
<td>GJ</td>
<td>Florida</td>
<td>1972</td>
<td>139</td>
<td>90</td>
<td>9.4</td>
</tr>
<tr>
<td>6088</td>
<td>California</td>
<td>1978</td>
<td>15</td>
<td>90</td>
<td>9.6</td>
</tr>
<tr>
<td>HB-5</td>
<td>Texas</td>
<td>1977</td>
<td>None</td>
<td>95</td>
<td>8.1</td>
</tr>
<tr>
<td>LEE(M-10)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Virginia</td>
<td>1968</td>
<td>51</td>
<td>100</td>
<td>14.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>There were 20 male DUB/ICR mice per group (13–18 g).

<sup>b</sup>Recorded to 28 days after inoculation.

<sup>c</sup>Mortality occurred with increased dosage.

<sup>d</sup>The M–10 denotes LEE strain amebae that had been serially mouse-passaged 10 times.

for the 13 strains was from 7.2 to 17.8 days. Kadlec (78) describes the great variation that occurred in virulence for 33 strains of *N. fowleri* isolated from the water of an indoor swimming pool. *Naegleria fowleri* amebae have been shown to differ in their susceptibility to trimethoprim, and the difference appears to be related to ameba virulence (22). The growth of virulent strains was unaffected by trimethoprim (400 μg/ml of medium), whereas avirulent isolates were completely inhibited by the drug at concentrations of 4 μg/ml. Trimethoprim susceptibility may be a useful aid in the selection of virulent environmental isolates of *N. fowleri*.

Ameba virulence appears to be related to the length of time a strain has been maintained in axenic culture (61). Axenic cultivation gradually decreases the virulence of *N. fowleri*. However, virulence can be enhanced and perhaps restored to original levels by passage of amebae in mammalian cell culture and by serial mouse passage (43, 142).

**Resistance and Susceptibility**

The factors responsible for innate resistance or susceptibility to naeglerial infection are undefined. Relatively few human infections have occurred even though large numbers of individuals have been exposed to similar environmental conditions. Most cases of PAM have occurred in previously healthy children or young adults.
When examining naeglerial infection in mice, it is readily apparent that the age of the test animals dramatically affects the outcome of *N. fowleri* exposure. Young mice are uniformly susceptible, but as they become older they also become increasingly more resistant to infection. Female mice have been shown to be more resistant to naeglerial infection than male mice of the same age and strain (60). Interestingly, there have been more male than female victims of PAM. This generally has been attributed to the vigorous swimming and diving and adventuresomeness of males and, thus, the probability of greater exposure to *N. fowleri* rather than to the possibility of greater susceptibility. Perhaps males, indeed, are more susceptible to infection, and specific hormones (or hormone levels) are involved. To date this area of research has not been investigated.

Different mouse strains vary greatly in their susceptibility to infection with *N. fowleri* (43, 60). In both these studies, C57Bl mice were the most resistant of the strains tested. One especially susceptible mouse strain (A/-HeCr) is deficient in the C5 component of complement (60). Obviously, it is premature to suggest that complement is involved in host resistance. Nonetheless, *N. fowleri* has been shown to be an efficient activator of serum complement by either the classical or alternative pathways (65, 106).

Serum obtained before death from a victim of PAM was assayed for immunoglobulin (Ig) levels (38). Although IgG and IgM levels were within normal limits, the IgA value was low. However, a second report from England (11) revealed normal levels of IgA for serum taken from a patient on day 3 of a fatal infection. In both patients, serum was measured, not secretory IgA levels. Because serum IgA levels may not be an accurate reflection of secretory IgA concentrations, both patients may have had deficient levels of secretory IgA. Serum IgA levels are greatly increased in mice given three intranasal inoculations of living *N. fowleri* (62, 72).

**Immunization and Protection**

Mice have been immunized with living, formalinized, and freeze-thawed *N. fowleri* via subcutaneous, intraperitoneal, intravenous, and intranasal routes of inoculation (74, 128). Protection of mice against lethal intranasal or intravenous challenge ranged from 6 to 55%. Intravenous immunization with *N. gruberi* afforded 65% protection (74), and three intranasal immunizing doses with *N. gruberi* produced 88% protection against intranasal challenge with *N. fowleri* (71). Nonpathogenic *N. gruberi* appears to be a better immunogen than *N. fowleri*. Perhaps man's unwitting exposure to *N. gruberi*, abundant in freshwater environments, affords protection against what normally would be a lethal exposure to *N. fowleri*.

Modest protection has been achieved by intraperitoneal immunization of mice with *N. fowleri* culture supernatant (124). Controls, receiving equal
volumes of fresh culture filtrate, did not exhibit protection. Presumably, protection was afforded by antigenic material derived from the amebae during cultivation. To date, all immunization attempts have produced only incomplete protection; solid immunity has not yet been achieved.

The mechanisms for protective immunity only recently have been given consideration. Protection against naeglerial infection can be transferred in mice by immune serum but not by sensitized spleen cells (125). Antibody to *N. fowleri* has been detected in surveys of normal human sera. Using a radioimmunoassay, the response against intracellular antigens was higher than the response against cell surface antigen (123). The indirect fluorescent-antibody test demonstrated titers ranging from 1:5 to 1:20 for 93 serum samples tested (35).

The role of cell-mediated immunity has been partly examined. A delayed hypersensitivity reaction has been described for guinea pigs sensitized by the soluble antigens of freeze-thawed *N. fowleri* (46) and by freeze-thawed amebae plus complete Freund adjuvant (142). Macrophage inhibition by the lymphokine macrophage inhibition factor has been described for *N. fowleri* (36).

*Naegleria fowleri* amebae have been shown by immunofluorescence to cap and remove or internalize surface-bound antibody (54). The ability of *N. fowleri* to remove antibody from the cell surface may enable the amebae to counter the host's immune defenses.

Neutrophils from *N. fowleri*-immunized mice are capable of killing amebae (55). One method of killing is for a group of neutrophils to surround an ameba and destroy it, presumably by contact and release of enzymes onto the ameba cell membrane. However, a novel phagocytic process has been described in which neutrophils pinch off portions of an ameba. Although unable to phagocytose an entire ameba, several neutrophils are able to rupture an ameba by pinching off and engulfing portions of it (55).

**CONCLUDING REMARKS**

Primary amebic meningoencephalitis, a fatal human disease caused by *Naegleria fowleri*, is known only as a somewhat rare or exotic disease, in short, a medical curiosity. However, circumstantial evidence suggests that human meddling with water resources may exaggerate the problem. If pollution and/or treatment of water increases the population of *N. fowleri* in public waters, then man and animals dependent upon impounded waters may be at serious and growing risk.

The determinative factors in virulence and host resistance to naeglerial infections are unclear in both human and experimentally induced PAM. Relatively few human infections have occurred, even though large numbers
of individuals have been exposed to similar environmental conditions. And most of these infections have occurred in previously healthy children or young adults. It is not known whether the higher incidence of disease among young males reflects behavioral attributes (adventurous, vigorous swimming and diving) or physiological factors (hormone levels, immunological competence).

The virulence factors that contribute to the pathogenesis of \textit{N. fowleri} are largely undefined. In vitro studies have implicated toxins and cytopathic enzymes, infectious cytopathogenic material, and phagocytosis, a natural function of all amebae. Perhaps it is not unreasonable to expect all of these factors to be involved in pathogenesis within the infected host.

Two recent cases of fatal meningoencephalitis resulting from free-living amebae have been described in which the organisms could not be identified positively as either \textit{Naegleria} or \textit{Acanthamoeba} \cite{50, 94}. The genus \textit{Vahlkampfia} has been suggested as an alternative. The significance of these two cases is that given the right conditions, serious disease in man (and possibly animals) may be produced by free-living amebae other than those we have come to regard as pathogenic.

\textbf{Acknowledgments}

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