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Biology of *Naegleria* spp.

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INTRODUCTION

Definition of the Genus and Its Species

The genus *Naegleria* consists of free-living amoebae widely distributed in soil and freshwater habitats throughout the world. These organisms characteristically undergo transformation from amoebae to flagellates. There are six species of *Naegleria*: *N. fowleri*, *N. australiensis*, *N. lovaniensis*, *N. gruberi*, *N. jadini*, and *N. thorntoni*. Each species may be distinguished on the basis of cyst morphology (163, 164, 199–201, 203), temperature tolerance (91), immunologic criteria (221, 234, 235), pathogenicity (17–19), and isoenzyme patterns (51, 53, 55, 94, 111, 155, 167–169). *N. gruberi*, a nonpathogenic species, was the first member of the genus to be described and has been used extensively to define the molecular biology of cellular differentiation (83). Another species, *N. fowleri*, sometimes called *N. aerobia* (133, 199, 201) or *N. invades* (27), is the causative agent of primary amoebic meningoencephalitis (PAME), a rapidly fatal disease of the central nervous system (CNS) (17–19). *N. australiensis*, originally isolated from flood waters in Australia, like *N. fowleri*, is pathogenic for mice, but the two species are antigenically and biochemically distinct (52). The pathogenicity of the two species for mice also differs: *N. australiensis* causes a subacute infection with focal meningoencephalitis, with fewer amoebae being present in the infected CNS tissue when compared with *N. fowleri* infection (54). Highly pathogenic strains of *N. australiensis* have been reported in Europe. The subspecies epithet *N. australiensis* subsp. *italica* has been proposed for these highly pathogenic strains (56). *N. lovaniensis*, a thermophilic environmental isolate, exhibits antigenic relatedness to *N. fowleri* but is not pathogenic for mice (205). *N. jadini*, isolated from swimming-pool water, is antigenically distinct from other species of *Naegleria* and is not pathogenic for mice (236). *N. thorntoni* was classified as *Didascalus thorntoni* by Singh (199) based on the pattern of nuclear division and the absence of an interzonal body. Page (163, 164) placed this species in the genus *Naegleria*.

Human disease caused by free-living amoebae was first reported in 1965 by Fowler and Carter, who studied four patients with PAME in South Australia (79). Subsequently, accounts of patients with PAME from the United States were reported by Butt in Florida (12, 13) and by Patras and Andujar in Texas (165). A retrospective study conducted by dos Santos (66) indicated that an epidemic of PAME had occurred in Richmond, Va., from 1951 to 1952. At the present time, episodes of PAME in humans have been reported from almost every continent (148, 233). The term "primary amoebic meningoencephalitis" was used first by Butt (12), and later by Carter (17), to distinguish infection of

the CNS in humans by free-living amoebae such as *N. fowleri* from the rare invasion of the brain by the intestinal amoeba *Entamoeba histolytica*. *E. histolytica* is a "true" parasite which may produce liver, lung, or brain abscesses from a primary focus in the colon (150). In contrast. *N. fowleri* is a free-living amoeba, which under unknown conditions becomes an opportunistic pathogen. PAME is a rapidly fatal disease which occurs generally in previously healthy children and young adults with a history of swimming in freshwater lakes or ponds. Presumably, infection results from introduction of water containing amoebae into the nasal cavity and subsequent passage of the organisms to the CNS via the olfactory apparatus (17–19). Acute hemorrhagic necrotizing meningoencephalitis follows invasion of the CNS. A review of the epidemiology, diagnosis, pathological aspects, and treatment of free-living amoebic infections has been published by Martinez (148). Other free-living amoebae, in soil and water, such as *Acanthamoeba* spp., are capable of producing a fatal CNS disease. Culbertson and his colleagues in 1958 were the first to establish the pathogenic potential of *Acanthamoeba* spp. (37–40). Organisms, identified as cell culture contaminants, were inoculated into monkeys and mice and produced amoebic encephalitis. Based on these studies, Culbertson predicted the possibility of human infection by free-living amoebae. Amoebic encephalitis produced by *Acanthamoeba* spp. is a chronic subacute disease known as granulomatous amoebic encephalitis (148) which can occur in chronically ill debilitated individuals, especially those with impaired immunity (145, 146). The incubation period for granulomatous amoebic encephalitis varies from 10 days to several weeks. Clinical disease is typified by abnormal neurological signs. Acanthamoebae have been isolated from eye lesions of human subjects with chronic corneal ulceration and from chronic ulcerations of the skin in addition to the CNS (148). *Acanthamoeba* spp. are antigenically distinct from both *Naegleria* spp. and *Entamoeba* spp. (221, 234). The biology of *Acanthamoeba* spp. has been reviewed by Schuster (189) and Martinez (147, 148).

Distribution in Nature

Naegleria spp. have been isolated from samples obtained from chlorinated swimming pools (21), freshwater lakes (67, 93, 107, 119, 230, 231), thermal springs (10, 184), domestic water supplies (3), thermally polluted waters (58–60, 65, 110), sewage (202), soil (10, 200, 203, 230, 231), air (117), humidifier systems (151), cell cultures (161), and human throat and nasal cavities (26, 30, 128). Soil is the preferred habitat of free-living amoebae, providing a source of oxygen, water, temperatures compatible with survival, and a bacterial food supply (200, 203). Runoff from heavy rains results in introduction of amoebae from soil into lakes and ponds. Such amoebae can be isolated from water samples by centrifugation and filtration (3, 41, 92, 93, 103, 119).

The vertical distribution of potentially pathogenic free-living amoebae in freshwater lakes and ponds has been correlated with physical, chemical, and biological parameters. Kyle and Noblet (119) noted that significant numbers of amoebae were isolated from water layers containing filamentous cyanobacteria and other eubacteria, both of which can serve as food sources for amoebae. *N. fowleri*, for example, was consistently isolated from particulate layers or detrital layers in lake water (120, 121). The largest number of *N. fowleri* isolated from a water column of such lakes originated from an attenuation zone containing exogenous iron (119). In a study in Virginia, the greatest number of pathogenic *Naegleria* strains were isolated from lakes that contained high concentrations of iron and manganese (67). *Naegleria* spp. apparently tolerate wide ranges of pH in the water; pH does not appear to be a factor limiting their distribution (60, 119).

Pathogenic *Naegleria* strains have been isolated from water at 10°C (58), pond mud at 16°C (230, 231), and soil at 8°C (10). Much attention has been directed toward isolating *Naegleria* spp., especially *N. fowleri*, from thermally polluted waters and from waters otherwise manipulated by humans (e.g., chlorinated water). *N. fowleri* has been isolated most often from thermal effluents, hot springs, and waters with naturally or artificially elevated temperatures (10, 58–60, 65, 110, 230, 231). The bacterial flora of the water appears to affect the incidence of *Naegleria* spp. Waters contaminated with coliforms have a high incidence of *Naegleria* spp. (10). *N. fowleri* has been isolated from tap water and from the domestic water supply in South Australia. The *Naegleria* amoebae found in these waters were implicated in a fatal case of PAME. This episode served as a model to explain infection of humans by methods other than swimming, such as face washing or bathing (3). Another human case of PAME, which occurred in a Nigerian farmer, involved the sniffing of water into the nasal passages as part of ritualistic washing before prayer. The water originated from a manmade pond at a farm (129).

Environmental samples, particularly those from heated waters of >40°C, often contain thermophilic nonpathogenic *N. lovaniensis* growing in competition with pathogenic *N. fowleri* (205). Flow cytometric techniques for rapidly identifying and quantitating species of pathogenic and nonpathogenic amoebae in environmental samples have been reported (152). Rapid identification of environmental isolates of *Naegleria* spp. by an API ZYM system (Analytab Products), which detects enzyme activity, also has been described. Differences in the patterns of activity of acid phosphatase and leucine arylamidase effectively differentiate *N. fowleri*, *N. lovaniensis*, and *N. australiensis* (112).

Soil amoebae may serve as vectors for other microorganisms. One of the more novel associations involves *Naegleria* spp. and *Legionella pneumophila*. Generally, *N. lovaniensis* destroys *L. pneumophila* within 24 h following ingestion. However, some amoebae become chronically infected and support the growth of *L. pneumophila*. *N. lovaniensis* does not become pathogenic as a result of ingestion of *L. pneumophila* (218). *N. fowleri*, which is pathogenic in humans, has been shown to interact differentially with *L. pneumophila* depending on the culture medium used. This amoeba is destroyed by *L. pneumophila* when in Page amoeba saline but not in amoeba culture medium. Newsome et al. (158) suggested that *Legionella* sp. retains its virulence in the environment because of its association with *Naegleria* spp. Amoebae full of *Legionella* sp., rather than free *Legionella* sp., may be the infective form for humans (181). *N. gruberi* has been shown to harbor viruslike particles (VLPs). These particles have been observed in the EGs strain of *N. gruberi* grown concomitantly with living bacteria but not in axenically grown amoebae (191). The bacteria apparently are not the source of VLPs since bromodeoxyuridine induces VLP formation in axenic cultures. These observations indicate that VLPs are present in a latent form within the nuclei of *N. gruberi* EG (190). In addition to VLPs, intracellular diphtheroids have been observed in *N. fowleri* (CJ strain). Antibiotic treatment eliminates these symbionts, but growth of the amoebae proceeds more rapidly in the presence of the diphtheroids (170). It has been reported that *N. australiensis*

grown with gram-negative bacteria is more pathogenic for mice than when grown axenically (52, 54). Conceivably the bacterial endotoxin or lipopolysaccharide in the amoebic preparations alters susceptibility of the host to PAME.

Naegleria spp. have been infrequently isolated from animals other than those from an aquatic environment. For example, *Naegleria* spp. have been isolated from the surfaces of aquatic or amphibian animals and from the intestines of fish (80, 210). In addition, antibodies specific for *Naegleria* species have been identified in certain animals (24). We observed that antibody to *N. fowleri* is widespread among dogs, swine, and sheep, but not cats. Because these animals occupy overlapping niches, these results indicate selectivity in exposure or responsiveness to naeglerial antigens. Selectivity in exposure may be related to the fact that *Naegleria* cysts can be transmitted by dust particles, especially in windy or arid environments. At least one infection attributed to inhalation of particles containing cysts occurred in Zaria, Africa (128). Indeed, the observation that children develop antibodies to *Naegleria* spp. in their toddling stage points to soil and vegetation, rather than impounded water, as the source of *Naegleria* spp. (178). Serological tests have been used extensively for the identification and classification of pathogenic and nonpathogenic amoebae from environmental isolations and from human brain isolations (2, 25, 27, 41, 43, 235).

GROWTH AND NUTRITION

Growth Requirements

Naegleria species are often cultivated in mixed cultures containing bacteria such as *Klebsiella* sp. or mammalian target cells. In addition, *Naegleria* spp. can be grown axenically in complex media. *N. fowleri* can be cultivated axenically in Nelson medium, consisting of Page amoeba saline, glucose, liver digest, and serum (154, 226). Similarly, *N. gruberi* can be cultivated axenically in Balamuth medium, which contains complex nutrients such as proteose peptone and yeast extract (5). In the presence of hemin, whole serum is not required for growth of the amoebae. We have observed that *N. fowleri*, *N. gruberi*, *N. lovaniensis*, and *N. australiensis* can be readily cultivated in Cline medium, which is based on Balamuth medium and Nelson medium supplemented with hemin and calf serum (34, 140). Cline medium contains 0.55% liver digest, 0.30% glucose, 0.50% proteose peptone, 0.25% yeast extract, 1% calf serum, and 1 μg of hemin per ml in Page amoeba saline.

Most media found suitable for axenic cultivation of both pathogenic and nonpathogenic *Naegleria* spp. contain proteose peptone, yeast extract, liver extract, or casein derivatives supplemented with bacteria, serum, or hemin (5, 20, 34, 153, 161, 226). A chemically defined minimal medium for the cultivation of *N. fowleri*, *N. lovaniensis*, and *N. australiensis* has been described (156). The medium consists of 11 amino acids (arginine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tryptophan, and valine), 6 vitamins (biotin, folic acid, hemin, pyridoxal, riboflavin, and thiamine), guanosine, glucose, salts, and metals.

The requirement of *N. fowleri* for iron and iron-containing compounds both in vitro and in vivo has been well documented. Exogenous iron greatly enhances the viability of *Naegleria* spp. in vitro (159). *N. lovaniensis*, *N. gruberi*, and *N. fowleri* ingest erythrocytes in vitro (1, 8, 37, 149). Indeed, it has been proposed that *N. fowleri* actively ingests host erythrocytes as their major source of nutrient, assimilating and using heme compounds (74). This concept is supported by the observation that deferrioxamine B and rhodotorulic acid, iron-chelating agents of microbial origin, exert a pronounced inhibitory effect on pathogenic *N. fowleri*. This inhibition can be diminished by adding iron to the chelators prior to incubation with amoebae (160).

Naegleria spp. are best grown in agitated cultures. Cultivations with a gyratory shaker have yielded large quantities of amoebae (i.e., 3×10^9 amoebae per liter) (226).

Physiology and Metabolism

Naegleria spp. are thought to be highly aerobic and reportedly do not grow in an anaerobic atmosphere of 90% N_2–10% CO_2 (226), although *N. fowleri* has been isolated repeatedly from anaerobic sediments (230, 231). *N. gruberi*, however, utilizes O_2 at a higher rate than *N. fowleri* (225). Using a defined minimal medium, Nerad et al. (156) noted that glucose, although metabolized in small amounts, was essential for growth of *Naegleria* spp. Growth of *N. fowleri* is accompanied by a pH increase in the medium, possibly from deamination of amino acids as preferred substrates (226). *N. fowleri* exhibit greater growth when lipids are present in the growth medium (98).

Several classes of digestive enzymes, including proteases, lipases, phosphatases, and esterases, have been identified in *Naegleria* spp. (169). Enzymes involved in the process of phagocytosis and intracellular digestion, such as lactate dehydrogenase, pyruvate kinase, catalase (239), and acid phosphatase (55), have been reported in highly pathogenic substrains of *N. fowleri*. High levels of a number of acid hydrolases have been demonstrated in *N. fowleri* amoebae (162). Enzymes also are apparently secreted by amoebae. Phospholipolytic enzymes which degrade sphingomyelin have been detected in the culture medium of *N. fowleri* (29, 100). These enzymes may be associated with pathogenicity. Pathogenic *N. fowleri* reportedly produce more phospholipase A and lysophospholipase than nonpathogenic *Naegleria* spp. (44, 100). Studies with whole-cell extracts of trophozoites have allowed for definition of the cellular compartmentation of hydrolytic enzymes. In *N. fowleri*, acid proteinase, *N*-acetylglucosaminidase, and acid phosphatase have been found to be associated with cytoplasmic granules resembling lysosomes; 5′-nucleotidase, with the surface membrane; aspartate aminotransferase, with mitochondria; and α-D-glucosidase and aminopeptidase activities, with both the surface membrane and lysosomal particles (132). Partial purification and characterization of *N. fowleri* β-glucosidase have been achieved (49). The enzyme has an apparent molecular weight of 66,000 and a pH optimum of 4.5 and is susceptible to inactivation by heat (67°C, 7 min). The β-glucosidase activity appears also to possess β-D-galactosidase activity. We have characterized an intracellular neutral aminoacyl-peptide hydrolase from *N. fowleri* (142). The exopeptidase activity is considered an aminopeptidase because the substrates leucyl naphthylamide and arginyl naphthylamide are hydrolyzed. The pH optimum of the exopeptidase is between 7.0 and 8.0. The aminopeptidase activity is impaired by cysteine protease inhibitors, e.g., iodoacetate and *p*-chloromercurisulfate, and the metalloprotease inhibitor *o*-phenanthroline. The *N. fowleri* enzyme is relatively heat stable and is not denatured by most detergents. The aminopeptidase may hydrolyze peptides to liberate free amino acids that may be used for biosynthesis. Alternatively, the aminopeptidase may process nascent pro-

teins. Numerous other enzymes have been detected in *N. fowleri*, *N. australiensis*, *N. lovaniensis*, and *N. gruberi*, although they have been neither purified nor characterized. These include malic enzyme, β-hydroxybutyrate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, L-threonine dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, superoxide dismutase, hexokinase, phosphoglucomutase, uridine diphosphate-glucose pyrophosphorylase, β-*N*-acetylglucosaminidase, aldolase, and glucose phosphate isomerase (169).

Susceptibility to Antimicrobial Agents and Antibiotics

The polyene antibiotic amphotericin B is amoebicidal, based on susceptibility testing in vitro, and has been regarded as the drug of choice for treating PAME (18, 19, 195). Amphotericin B has been shown to produce changes in nuclear shape and mitochondrial abnormalities, to increase proliferation of both the rough and smooth endoplasmic reticula, to decrease the number of food vacuoles, and to increase formation of autophagic vacuoles in the amoebae. This antibiotic also has been shown to inhibit pseudopod formation and to induce blebbing of the amoeba plasma membrane (192). Clotrimazole exhibits amoebicidal activity in vitro but has no effect in vivo (102). Trimethoprim, an antagonist of folic acid metabolism, reportedly inhibits the growth of nonvirulent *N. fowleri* isolates but has no effect on the growth of virulent strains (23). The susceptibility of *N. fowleri* and *N. gruberi* to antibiotics and antimicrobial agents has been shown to be influenced by the composition of the nutrient medium in which the amoebae are cultivated. For example, *N. fowleri* grown in Nelson medium is more resistant to actinomycin D, daunomycin, mithramycin, sulfamethoxazole, or tyrocidine than when grown in Balamuth medium (34). These data indicate that comparative studies of different species or strains of *Naegleria* should be performed on amoebae grown in the same nutrient medium. Δ^9-Tetrahydrocannabinol, the major psychoactive component of marihuana, inhibits the growth of *N. fowleri* when used at high concentrations (25 to 50 μg/ml). The cannabinoid also prevents enflagellation and encystment of the amoeba, but has no effect on amoeboid movement (175, 176).

Destruction by Chemicals

Studies on the effects of free chlorine and elemental I_2 on *Naegleria* spp. have indicated that the two halogens have similar cysticidal activity. Free chlorine at 0.5 μg/ml readily kills *Naegleria* cysts, while lower doses are known to destroy the trophozoite stage. *N. fowleri* trophozoites and cysts are more sensitive to chlorine than *N. gruberi* (57, 183). Above 3.4 μg/ml, I_2 is more cysticidal than is free chlorine (28). The comparative amoebicidal capacities of chlorine, chlorine dioxide, ozone, Deciquam 222, and Baquacil (polyhexamethylene biquanide hydrochloride) have been assessed. All five disinfectants possessed amoebicidal activity which was dependent on the chemical and physical characteristics of the water to be treated (45, 50). The amoebicidal activity of chlorine on *N. gruberi* cysts is effectively inhibited by cyanuric acid (73).

The effects of various physical factors on the growth and survival of *Naegleria* spp. have been studied. *Naegleria* spp. tolerate a relatively wide pH range (4.6 to 9.5) in vitro (18, 119). *Naegleria* amoebae also possess a higher temperature tolerance range than most other free-living amoebae. Strains of *N. fowleri* isolated from human infections grow readily at 45°C (91, 92). The amoebae and cysts can tolerate higher temperatures (i.e., 65°C) for short periods of time (1 and 3 min) (28). Cerva demonstrated that temperatures below 20°C were inhibitory to the in vitro multiplication of *N. fowleri* (22). Trophozoites of *N. fowleri* have been reported to degenerate at temperatures below 10°C (28).

N. fowleri does not tolerate salinity as well as amoebae which do not form a flagellate stage. Most strains of *N. fowleri* tolerate 0.5% to 1% NaCl (93). However, growth of some strains of *N. fowleri* is inhibited by 0.2% NaCl and KCl. $CaCl_2$ stimulated encystment of *N. fowleri* (109).

Preservation

Drying has a rapidly lethal effect on *N. fowleri* trophozoites and cysts. In contrast, *N. gruberi* cysts are viable upon rehydration 23 months after drying. Cysts of *N. fowleri* were rendered nonviable by lyophilization, whereas those of *N. gruberi* remained viable after 6 months in a lyophilized state (28). Storage temperature has a major effect on survival of *Naegleria* spp. Cysts of *N. fowleri* stored at −30°C were nonviable compared with those of *N. gruberi*, which had a 4-month survival time when stored at −25 to −30°C. *N. fowleri* and *N. gruberi* cysts remained viable for 8 months at 4 or 15°C (6, 224). At room temperature, *N. fowleri* and *N. lovaniensis* cysts incubated with *Enterobacter cloacae* on Page amoeba saline agar slants were viable for 6 months, while *N. gruberi* was viable for up to 24 months. Storage of trophozoites in the presence of 5% dimethyl sulfoxide in liquid nitrogen results in a 63.3% recovery of *N. fowleri* and a 48.8% recovery of *N. gruberi* (197).

STRUCTURE AND FUNCTION

Structure of Trophozoites

The trophozoite of *Naegleria* is the vegetative or feeding stage. Trophozoites exhibit food cups or amoebastomes, which are cytoplasmic extensions of the surface (Fig. 1A). The food cups, which vary in size and number depending on the species and strain of *Naegleria*, are used to ingest bacteria, yeast cells, and cellular debris (Fig. 1C and D) (105, 106, 127, 140, 141). They also may serve as attachment organelles (140, 141). The more virulent strains of mouse-passaged *N. fowleri* exhibit fewer food cups than do axenically grown *N. fowleri* (Fig. 1B). The nonpathogenic species of *Naegleria*, *N. lovaniensis* and *N. gruberi*, occasionally exhibit small food cups (127, 140). These observations indicate that the presence of food cups does not correlate with pathogenicity (140).

At the ultrastructural level, *Naegleria* trophozoites exhibit typical features of a eucaryotic cell (Fig. 2A and B), with the exception that centrioles have yet to be observed (86, 185). The amoeba is surrounded by a unit membrane (10 nm in width) composed of two dense layers enclosing a clear space (185). Large numbers of ribosomes, both free and attached to the endoplasmic reticulum, are found scattered throughout the cytoplasm. A smooth endoplasmic reticulum is also present. Although a typical Golgi apparatus has not been observed, a "primitive" Golgi-like complex, made up of membranous components and coated vesicles, has been identified in some strains of *N. fowleri* (206) and in *N. lovaniensis* (205). Numerous membrane-bound cytoplasmic organelles have been observed in *Naegleria* spp. (166). The mitochondria observed in these amoebae are dumbbell or

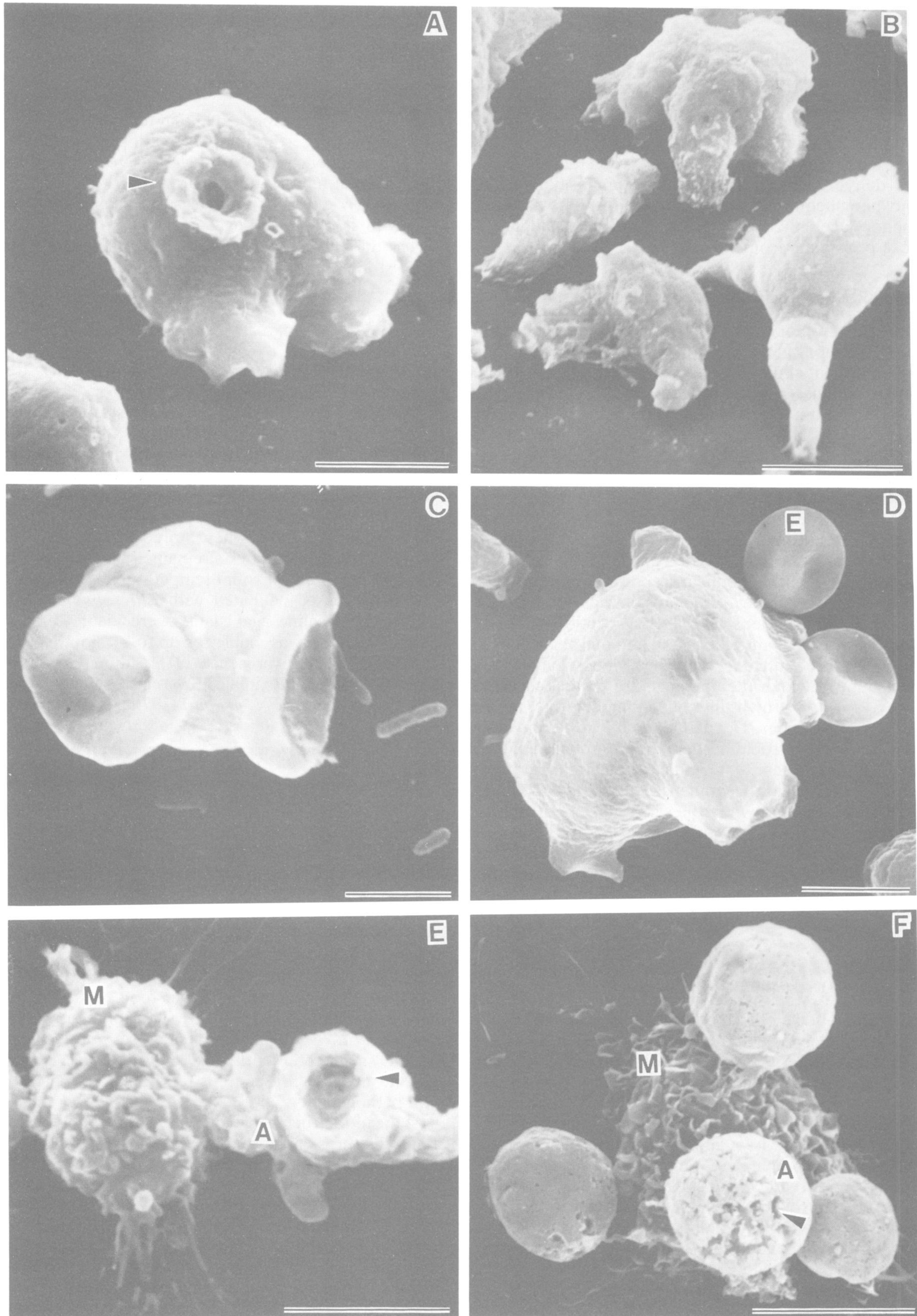

FIG. 1. Scanning electron micrographs of *N. fowleri* amoebae. (A) *N. fowleri* LEE grown axenically in Nelson medium with a single food cup or amoebastome (arrow). (B) Highly pathogenic mouse-passaged LEEmp; note the absence of food cups. (C) LEE in the presence of bacteria; prominent food cups are used to ingest bacteria. (D) LEE in the presence of human erythrocytes (E). (E) LEE amoeba (A) attached to a resident macrophage (M). Arrow indicates food cup. (F) LEE amoebae (A) attached to a BCG-activated macrophage (M). *N. fowleri* lose their amoeboid appearance and food cups are not present. Arrow indicates damage to amoeba membrane. Bar, 5 μm.

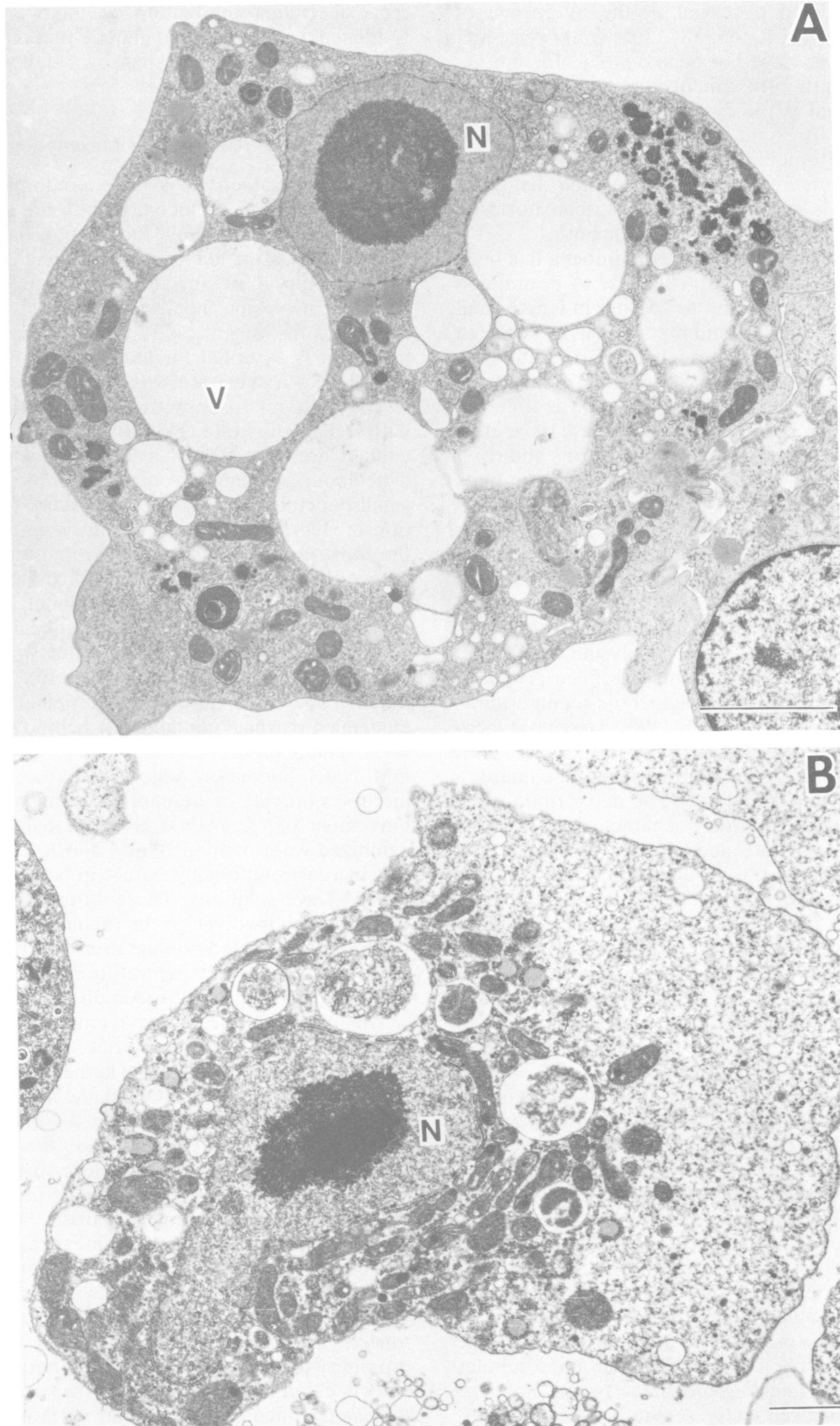

FIG. 2. Transmission electron micrographs of trophozoites of *Naegleria*. (A) *N. australiensis* (PP397). (B) *N. lovaniensis* (Aq/9/1/45D). N, Nucleus; V, vacuole. Bar, 5 μm.

cup shaped (16, 166, 185, 189). Studies performed with intact and functional mitochondria, from both *N. fowleri* and *N. gruberi*, have indicated that oxidation is tightly coupled to phosphorylation (228, 229). Primary and secondary lysosomes, identified by acid phosphatase staining, are dispersed in the cytoplasm (74). Contractile vacuoles also have been identified in the tail region or "uroid" of *Naegleria* spp. (185, 219). Membrane-bound black bodies, 40 to 80 nm in

diameter, also have been observed in the cytoplasm of *Naegleria* amoebae (16, 133, 166, 185, 205, 206). The functions of these structures have not been defined. In addition to membrane-bound structures, inclusions not limited by a membrane are localized in the cytoplasm. Globular bodies, termed lipid globules by some investigators (149, 220) and electron-translucent droplets by others (166), dominate the cytoplasm of *N. fowleri*. Feldman (74) postulated that these globular bodies serve as storage organelles or depositories of inactive lysosomal hydrolases or catalase or both.

The cytoskeletal structure of *Naegleria* amoeba has been examined by various investigators. Thick (17 to 19 nm) and thin (5 to 7 nm) microfilaments up to 256 nm in length, and morphologically similar to actin and myosin, have been seen in the cytoplasm of *N. fowleri* amoebae (125). Actin, the major polypeptide synthesized by *N. gruberi*, has been purified to homogeneity (209). *N. gruberi* actin is different from actin isolated from other eucaryotes because (i) isoelectric focusing resolves three distinct actin isoforms and (ii) it lacks *N*-methylhistidine. Antibodies prepared in rabbits against purified *N. gruberi* actin are specific to *Naegleria* actin and do not recognize determinants in actin from other amoeboid organisms such as *Acanthamoeba* or *Dictyostelium* spp. (88). The thin microfilaments are concentrated near the plasma membrane, while the thick microfilaments are randomly oriented in the cytoplasm. These latter structures do not appear to be attached to the plasma membrane. Interaction between the microfilaments has been proposed as the mechanical means by which amoeboid locomotion is accomplished in *Naegleria* spp. (125, 126). Amoeboid locomotion is perturbed by the addition of cytochalasin B (114).

The nucleus of *Naegleria* spp. is prominent and contains a conspicuous nucleolus or endosome. The deoxyribonucleic acid of *N. gruberi* has been partially characterized and was found to be rich in adenylate and thymidylate (134). The nuclear envelope is typical for eucaryotes and contains numerous pores. The outer membrane of the nuclear envelope of *N. fowleri*, *N. gruberi*, and *N. jadini* is coated with ribosomes (16, 205, 206). In contrast, ribosomes on the outer membrane of the nuclear envelope are absent in *N. lovaniensis*, but several parallel stacks of cytoplasmic rough endoplasmic reticulum circumscribe the nucleus. Extruded nuclear material may be present in the cytoplasm of *Naegleria* spp. and has been observed in *N. lovaniensis* trophozoites (205). The deoxyribonucleic acid content per amoeba during log growth phase is similar for both *N. fowleri* and *N. gruberi* (i.e., 0.3 pg per amoeba). The ribonucleic acid (RNA) content is approximately 18 pg per amoeba for *N. fowleri* and 8 pg per amoeba for *N. gruberi* (225, 227).

Nuclear division in *Naegleria* spp. is unusual in that the nuclear membrane remains intact throughout karyokinesis and the nucleolus persists. In the early stages of mitosis, enlargement of the nucleus and condensation of the chromosomal material occur, but distinct chromosomal elements are not apparent (86). The nucleus, then, elongates and the nucleolus becomes dumbbell shaped. Spindle microtubules appear in the nucleus, and chromosomes orient along an equatorial plate and subsequently separate. The nucleolus divides, resulting in the formation of large nuclear polar masses composed of RNA and protein. The interzonal body, containing nucleolar material, extends between these polar masses (87, 171, 177, 199, 201). Centrioles have not been observed in mitotic amoebae (86, 185). The elongated nucleus finally separates into daughter nuclei. Nuclear division, then, is followed by division of the cytoplasm.

Chromosome number and ploidy, as well as the occurrence of sexual reproduction, are unknown in *Naegleria* spp. Evidence for diploidy and genetic recombination in *Naegleria* spp. has been reported based on electrophoretic variation for 15 enzyme-coding genes (15).

Amoeboid Locomotion

Naegleria amoebae display a predominantly monopodial "limax" pattern of locomotion (163, 219). Cell surface changes during amoeboid locomotion have been studied in *N. gruberi*, using fluorescent staining with conjugates of concanavalin A or specific antiserum. Such studies have shown that plasma membrane turnover is not required for amoeboid movement (173, 174). Adhesion to a substrate, however, is essential for locomotion. This dependence on adhesion to a substrate was recognized based on reflexion interference microscopy of the interaction of the amoebae with a glass substrate (113–116, 172, 173). In *N. gruberi*, the contact area consists of a large broad platform or "associated contact" region from which are suspended, ventrally, small punctate stable "focal contacts" (172). A direct relationship has been shown between closeness of the amoeba to the substrate and the speed of locomotion. Divalent, but not monovalent, ions reduce the cell-substrate gap and thus enhance locomotion. The movement of amoebae on a weakly adhesive versus a strongly adhesive glass surface has been investigated. In deionized water, *N. gruberi* develops a weak cell-substrate adhesion (113, 116). *Naegleria* amoebae moving in deionized water experience membrane loss as evidenced by focal contacts left behind on glass cover slips (113). The addition of small amounts of electrolyte, e.g., 10 mM NaCl, increases adhesion to the substrate and also increases the rate of locomotion of the amoebae. The rate of movement of *N. gruberi* is three to four times slower in deionized water than in 10 mM NaCl. The speed of locomotion increases with temperature in both deionized water and 10 mM NaCl solutions. These differences are thought to be due to forces involved in the maintenance or modulation of the broad associated contact area and not the focal contact area. An increase in temperature from 20 to 30°C leads to a doubling in the speed of locomotion (114).

The migration rates and movement of *Naegleria* amoebae have been examined by a variety of techniques (182). The migration rate of amoebae, whether thermotolerant or not, depends on temperature. On sparse nutrient agar with *Escherichia coli*, *N. fowleri* advances at 1 to 3 mm/day at 23°C, 7 to 14 mm/day at 37°C, and 7 to 18 mm/day at 43°C. *N. lovaniensis* migrates at a rate close to that of *N. fowleri* at 24°C (91–93). Four species of *Naegleria* have been tested for their ability to migrate under agarose. Pathogenic *N. fowleri* and the moderately pathogenic species *N. australiensis* exhibit more rapid locomotion than *N. lovaniensis* and *N. gruberi* at 37°C (213).

We have evaluated the response of the pathogen, *N. fowleri*, and several other species of *Naegleria* placed in proximity to cells of neural and non-neural origin. *N. fowleri* amoebae, but not those of *N. australiensis*, *N. gruberi*, or *N. lovaniensis*, demonstrate enhanced motility when placed in proximity to mammalian cells. Amoebae of nonpathogenic species of *Naegleria*, however, are more motile in Eagle minimal essential cell culture medium than the amoebae of *N. fowleri*. *N. fowleri* responds to disrupted nerve cells more vigorously than to disrupted Vero cells. The locomotory response of highly pathogenic mouse-passaged *N. fowleri* amoebae to nerve cells is greater than axenically cultured amoebae. The enhanced motility elicited by whole or dis-

rupted nerve cells is not directed migration but chemokinetic (33). However, *N. fowleri* responds chemotactically and chemokinetically toward live cells and extracts of *E. coli* and other bacterial species. Presumably, chemotaxis by *Naegleria* spp. would play an important role in locating a food source in the environment. Conditioned medium from *E. coli* cultures does not stimulate movement of *N. fowleri*. Toxic metabolic end products or exotoxins from bacteria possibly prevent migration and kill or repel amoebae (138).

The Flagellate Stage

When amoebae are incubated in non-nutrient buffer, they transform from feeding dividing organisms into transient nonfeeding nondividing flagellates. Differentiation into the flagellate stage involves a change in cell shape and a change in synthesis of all organelles of the flagellar apparatus. *N. gruberi* completes this transformation in about 120 min (83, 84); *N. fowleri* requires several hours. Ultrastructural changes related to the flagellate phenotype of *N. fowleri* are not discernible during the initial 90 min of subculture in non-nutrient buffer. However, by 2 h into the enflagellation process, the number of cytoplasmic vacuoles decreases, and basal bodies, a rootlet, and flagella are formed. The flagellar apparatus is partially developed in the amoeba before an obvious change in cell shape occurs. The rootlet extends into the cell perpendicular to the basal body, and the emerging flagellum becomes associated with the nucleus at the leading end of the cell. The rootlet comes to lie in a furrow or groove extending the length of the nucleus at the termination of the enflagellation process. Flagella of *N. fowleri* and *N. gruberi* exhibit the typical 9 + 2 arrangement of filaments surrounded by a sheath continuous with the cytoplasmic membrane (166, 185). The outer circle of flagellar doublets is continuous with the cylinder of nine triplet filaments which make up the basal body. The rootlet is connected to the basal bodies by an intricate series of parallel and transverse microtubules. Anchoring microtubules also are grouped around the basal bodies. The mature flagellar apparatus usually consists of two flagella, two basal bodies, microtubules, and a single striated rootlet or rhizoplast (64, 83).

The Cyst Stage

Cysts of *N. gruberi* and *N. fowleri* have been described by using both light (177, 186, 199, 200, 219) and electron (124, 186, 188, 219) microscopes. Differences in cyst structure have been used as a morphological criterion for discrimination of *Naegleria* species.

The cyst stage affords protection from desiccation and food deprivation. Electron microscopy has demonstrated differences in the cyst wall of *N. gruberi*, *N. fowleri*, and *N. jadini* (188, 206). The mature cyst of *N. gruberi* is encompassed within a double-walled structure about 20 μm thick which is thought to be composed of mucopolysaccharides (186). The outer layer of the cyst wall present in the cysts of *N. gruberi* is absent in the cysts of *N. fowleri* and *N. jadini*. *Naegleria* spp. exhibit pores in the cyst wall. A mucoid plug which seals the pores is thinner in *N. fowleri* than in *N. gruberi* cysts. The encysting *Naegleria* contains a nucleus and a variety of cytoplasmic vacuoles, including food vacuoles and contractile vacuoles (186, 188). During the early encystment period, the mitochondria enlarge and the cisternae of the endoplasmic reticulum become inflated. The cyst wall material is thought to be synthesized and packaged by the rough endoplasmic reticulum (206). The nucleus of the encysted amoeba contains a nucleolus, but the nucleolus is less pronounced than in the trophozoite stage (186, 188, 193). Ribonucleoprotein-containing vesicles, probably autophagosome-type vacuoles, have been observed in the cytoplasm of encysted amoebae (193). The wall of the *N. gruberi* cyst is not dissolved during excystment, and the cyst remains long after the trophozoite emerges (188).

DIFFERENTIATION

Enflagellation

Enflagellation normally occurs when amoebae are subjected to nutritional deprivation (237). Flagellates are temporary forms that eventually revert to amoebae. *N. gruberi* can be induced to enflagellate by brief exposure to high hydrostatic pressure (3,500 lb/in^2) (217). Temperature shock induces the formation of multiflagellated *N. gruberi* (63). The transformation of amoebae to flagellates that occurs when the amoebae are transferred from growth medium to distilled water can be suppressed by KCl, NaCl, LiCl, $CaCl_2$, or $MgCl_2$ (11).

Studies performed with *N. gruberi* amoebae have indicated that deoxyribonucleic acid synthesis is not required for transformation to the flagellate form (36, 144). However, transformation is dependent on transcription and translation (89, 122, 223). The RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide prevent differentiation if added shortly after cells are transferred to non-nutrient buffer (84, 89). When transformation from amoebae to flagellates begins, actin synthesis ceases and translatable actin messenger RNA (mRNA) disappears. During this period, new mRNAs, including those for flagellar tubulin subunits, appear (122, 208). Several proteins of the flagellar apparatus are synthesized de novo, including the flagellar rootlet and the basal bodies which result in the formation of the typical centriolelike structure of nine triplet microtubules. In contrast to other eucaryotic systems, the formation of the centriolelike structure does not require the presence of preexisting template centrioles (123). The tubulin which comprises the flagellar microtubules is synthesized de novo during the transformation process. This conclusion is supported by the observation that little or no flagellar tubulin is synthesized when the wheat germ cell-free protein-synthesizing system is directed by RNA extracted from amoebae prior to transformation (84, 118, 122). Flagellar tubulin has a subunit molecular weight of 55,000, which separates into α and β subunits when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both tubulin subunits apparently are synthesized simultaneously during differentiation since translatable mRNAs for the two subunits appear and disappear together. However, the α and β subunits are encoded on separate mRNAs. The mechanism by which this coordinated expression is regulated remains to be defined.

Mar et al. (135) investigated the nature of the requirement for RNA synthesis during differentiation of *N. gruberi* into flagellates by examining polyadenylated RNAs that were specific for differentiating cells. A complementary deoxyribonucleic acid library prepared from polyadenylated RNA extracted from amoebae 40 min after initiation of transformation was cloned into pBR322, and differentiation-specific plasmids subsequently were identified. Four classes of differentiation-specific RNAs appeared coordinately during differentiation. Changes in the concentration of all four classes

of differentiation-specific RNAs were observed to follow the temporal pattern of changes in levels of flagellar tubulin mRNA as monitored by the in vitro translation system. These studies indicate that changes in flagellar tubulin mRNA were the result of changes in the concentration of the RNAs and not changes in rates of translation.

The synthesis and formation of the flagellate cytoskeleton have been followed during transformation by immunofluorescence microscopy, using a monoclonal antibody, AA-4.3, which binds to the β-subunit of tubulin. This antibody recognizes a determinant common to flagella and cytoplasmic fibers. In flagellate-shaped cells, cytoskeletal microtubules were seen to radiate from the base of the flagella extending to the full length of the cell (222). Antibodies prepared against flagellar tubulin have been shown not to cross-react with tubulin present in amoebae. The striated rootlet or rhizoplast of *N. gruberi* which anchors the flagellar bases has been isolated. Purification of a major rootlet protein having an apparent subunit molecular weight of 170,000 has been achieved (123). Studies with antiserum raised against the rhizoplast have demonstrated that rhizoplast proteins are absent in amoeboid stages (90).

Calcium may have both a direct and an indirect role in the transformation process. Direct inhibition of transformation by calcium has been demonstrated with the ionophore A23187, amphotericin B, a substance with ionophorelike activity, or the hormone calcitonin, an agent which facilitates calcium uptake into the cytosol. An indirect role for calcium in inhibition of transformation due to a calcium-regulatory protein and its control of cyclic nucleotide levels in the cell has been proposed based on inhibition by trifluoperazine, a calmodulin antagonist (194). Changes in cyclic nucleotide levels occur during amoeba-to-flagellate transformation. Absolute levels of both cyclic adenosine 3′,5′-phosphate and adenosine 5′-triphosphate decrease during transformation (35). Two calcium-binding proteins are synthesized during differentiation and serve distinct roles in the flagellates. These proteins are calmodulinlike and differ in apparent molecular weight and intracellular location. The first of these is calmodulin 1 and is localized in flagella. This protein has an apparent molecular weight in the presence of calcium of approximately 16,000. The second calcium-binding protein is calmodulin 2 and is localized in cell bodies. This latter protein has an apparent molecular weight of 15,300. Since translatable mRNAs encoding calmodulins 1 and 2 increase and decrease concurrently with mRNAs for flagellar tubulins, it has been suggested that expression of the unrelated genes for calmodulin and tubulin may be under coordinate control during differentiation (84, 85).

The conversion of *N. fowleri* amoebae to flagellated cells occurs 150 to 180 min after cells are suspended in Page saline. Amoebae from the stationary phase of growth enflagellate more readily than actively growing amoebae. The stimulus for enflagellation is suspension, dilution, and agitation in non-nutrient buffer (240). Although there are many similarities in enflagellation of *N. fowleri* and *N. gruberi*, there are a number of differences in the regulation of this process. Both *N. fowleri* and *N. gruberi* flagellates revert rapidly to amoebae. The effect of temperature, however, is different. *N. gruberi* enflagellates readily when subcultured to the same or lower temperature (83, 84), whereas *N. fowleri* enflagellates with higher yields when subcultured to the same or higher temperature (240). Population density also influences enflagellation of *N. fowleri*. In contrast, cell density does not appear to be a critical variable for *N. gruberi* enflagellation. *N. fowleri* synthesizes many macromolecules during the enflagellation process as suggested by the observation that actinomycin D and cycloheximide inhibit differentiation. The addition of actinomycin D at early and late time periods arrests enflagellation of *N. fowleri* (240). In the case of *N. gruberi*, cycloheximide does not block differentiation if added at 16 to 20 min before cells form flagella (84, 122, 223). In contrast, cycloheximide stops differentiation of *N. fowleri* and accelerates the rate of reversion of flagellated cells to amoebae (240).

Analysis of polypeptide profiles of two-dimensional gels of whole-cell extracts of *N. fowleri* flagellates and growing amoebae have indicated that the levels of most proteins are relatively stable during enflagellation. However, a few polypeptides decreased in amount or vanished during enflagellation while other polypeptides increased markedly in concentration. A few new polypeptides appeared during enflagellation which were thought to be either modifications of preexisting proteins or newly synthesized polypeptides (241, 242). Flagellation-specific polypeptides were not detected. Woodworth et al. (242) have suggested that changes associated with differentiation in *N. fowleri* involve regulatory processes such as quantitative shifts, redistribution within the cell, or altered molecular interactions rather than extensive synthesis of unique proteins.

Encystment

In addition to forming flagellates, *Naegleria* amoebae form cysts. Factors thought to induce cyst formation include food deprivation, crowding, desiccation, accumulation of waste products, exposure to metabolic products of bacteria, lack of oxygen, pH changes, and salt concentration (27, 28, 200, 203). Encystment of *Naegleria* spp. entails cyst wall formation, expulsion of water by contractile vacuoles, and reorganization of the cytoplasm (189, 193). Although the exact conditions that induce encystment in the environment remain to be defined, cyst formation in the laboratory has been shown to occur in liquid axenic and bacteria-containing cultures. *N. fowleri* and *N. gruberi* grown in association with living *E. coli* form cysts (57). Cyst formation occurs also when *N. fowleri* are grown on agar supplemented with *Klebsiella aerogenes* (224). Certain chemicals have been shown to enhance encystment. For example, cyst formation of *N. fowleri* is promoted in medium containing 2×10^{-3} M $CaCl_2$ (109).

We have noted the existence of marked strain differences in cyst formation. *N. fowleri* LEE does not readily form cysts in axenic culture, but a highly pathogenic mouse-passaged substrain, LEEmp, encysts rapidly late in the stationary phase of growth. Other studies have shown that *N. fowleri* encyst when in close proximity to *Pseudomonas aeruginosa*, a nondigestible bacterium (138). This observation suggests that toxic metabolic products of bacteria may induce cyst formation.

Signals for excystment include addition of fresh culture medium, water (186), the presence of bacteria, and CO_2 (4). Cysts of some strains of *N. gruberi* excyst in response to elevated levels of CO_2, while other strains excyst when placed in a fresh aqueous environment such as water or medium. Averner and Fulton (4) reported that anything that causes an increase in CO_2 (i.e., bacteria, chemicals, or an increase in temperature) leads to excystment. Excystment of *N. gruberi* also has been induced by exposure of cysts to high hydrostatic pressure (217).

MECHANISMS OF CYTOPATHOGENICITY IN VITRO

Naegleria as the Effector Cell

Many investigators have reported that animal pathogenic strains of *N. fowleri* are cytopathic for cultured mammalian cells (7–9, 27, 37, 42, 72, 104, 140, 141, 143, 220). Conversely, other investigators have reported that the nonpathogenic species, *N. gruberi*, is not cytopathic for cultured mammalian cells, concluding that cytopathogenicity of *Naegleria* spp. is correlated with pathogenicity (27, 37, 42). However, nonpathogenic *Naegleria* spp. produce a profound cytopathic effect in vitro, and cytopathogenicity may not always correlate with pathogenicity in vivo (137, 140, 143, 243).

We have observed that pathogenic *N. fowleri*, weakly pathogenic *N. australiensis*, and nonpathogenic *N. lovaniensis* and *N. gruberi* produce cytopathic effects (CPE) in cultured mammalian cells in vitro (137, 140, 143). *N. australiensis*, *N. lovaniensis*, and *N. gruberi* lyse cultured nerve cells on contact. This lysis is probably not the result of toxic products or enzymes released into the medium, because not all cells are lysed simultaneously. Amoebae appear to ingest target cell constituents following lysis. We have noted also that amoebic extracts of the four *Naegleria* species produce CPE on B103 neuroblastoma cells in culture (140). The mechanism of cytopathic action of *Naegleria* spp., however, remains a matter of controversy. Chang (27, 28) suggested that CPE of *N. fowleri* were due to a cytolytic substance liberated by the amoebae. Visvesvara and Callaway (220) noted that CPE in Vero cell cultures was the result of a combination of phagocytosis and the cytolytic action of enzymes. Brown (7–9) concluded that *N. fowleri* injured target cells by repeated nibbling, a process he termed "trogocytosis." Delayed CPE has been shown to occur in the presence of *N. fowleri*-specific antiserum which immobilizes or agglutinates trophozoites. Total inhibition of CPE also has been obtained in the presence of cytochalasin B (8).

Naegleria-induced CPE also has been attributed to the transmission of an infectious cytopathogenic agent from trophozoites to susceptible mammalian cells (68–72). However, the cytopathology caused by the *Naegleria* amoeba cytopathogenic material is not attributed to VLPs (187, 191). It has been proposed that *Naegleria* amoeba cytopathogenic material is a protein that shares many features with infectious agents such as cell specificity, cell to cell spread, and amplification of "infectious" material upon passage through cells (72).

Recent studies in our laboratory indicate that major differences in the mechanism of cytopathogenicity occur between axenically cultivated *N. fowleri* (LEE) and a highly pathogenic mouse-passaged strain of *N. fowleri* (LEEmp). Transmission and scanning electron microscopy studies of B103 nerve cells inoculated with *N. fowleri* confirm that the cytopathology induced by axenically cultivated amoebae is a result of piecemeal ingestion of target cells (141, 143). The ingestion is not mediated by pseudopodial extensions of *N. fowleri*. Rather, a suckerlike apparatus called a food cup or amoebastome is utilized for ingestion of portions of the B103 nerve cell (Fig. 3A). In contrast, the highly pathogenic mouse-passaged strain of *N. fowleri* lyses B103 nerve cells (Fig. 3B). Our observations indicate that lysis of target nerve cells by mouse-passaged amoebae requires cell-cell contact and cytolytic factors apparently are not secreted into the culture medium.

Cytolytic Factors

We have investigated the cytopathic potential of cell-free lysates of *N. fowleri* to characterize the putative role of cytolytic substances associated with *Naegleria* spp. We used ^{51}Cr release to measure cytopathic activity of *N. fowleri* cell-free lysates for nerve cells. Specific release of chromium from radiolabeled target cells is considered to be an indication of damage to the labeled target cell plasma membrane. B103 nerve cells labeled with ^{51}Cr were used as indicator target cells to detect cytolytic factors obtained from *N. fowleri* (81). Cytolytic activity associated with axenically grown *N. fowleri* amoebae was recovered in a soluble fraction of cell-free lysates. Potentially cytotoxic enzymes such as phospholipase A, phospholipase C, and proteases are present in the soluble cytolytic fraction derived from cell-free lysates. Evidence has accumulated which implicates a phospholipase A enzyme as a cytolytic factor. Reduction of cytolytic activity has been obtained in the presence of the phospholipase A inhibitor Rosenthal's reagent (82). Phospholipase A activities of *N. fowleri* are greatly reduced by ethylenediaminetetraacetate (100) which, furthermore, greatly reduces the cytolytic activity of the *N. fowleri* soluble fraction (82). Chang (29) concluded that a phospholipolytic enzyme released by *N. fowleri* into the culture fluid during active growth was responsible for the CPE on primary kidney cell cultures. Cursons et al. (44) demonstrated that both pathogenic and nonpathogenic amoebae produced phospholipase A. Higher levels of phospholipase A were produced by pathogenic strains compared with low levels of the enzyme in nonpathogenic strains. Phospholipase A, sphingomyelinase, and lysophospholipase activities have been detected in amoeba culture medium and cell-free homogenates of *N. fowleri* (100). The ability of these phospholipases to degrade phospholipids of human myelin in vitro has been reported (101). Other factors may be present in the soluble fraction obtained from *N. fowleri* amoebae since Rosenthal's reagent fails to inhibit completely the cytotoxic activity. Indeed, phospholipase C activity also has been detected. Heat treatment (50°C, 30 min) of the soluble fraction reduces both phospholipase C activity and cytolytic activity but does not reduce phospholipase A activity. However, several cytolytic factors may be produced by *N. fowleri* because neither phospholipase A nor phospholipase C activities alone produce the maximum cytolytic effect (82). In a recent study of *N. fowleri* cytolytic factors, Lowrey and McLaughlin (130, 131) reported the partial characterization of hemolytic activity associated with cell-free lysates of *N. fowleri* trophozoites. The majority of hemolytic activity was associated with the surface membrane of *N. fowleri*. Chromatofocusing of *N. fowleri* whole-cell extract yielded three hemolytic peaks which displayed synergistic hemolysis. The hemolytic factors were not secreted by *N. fowleri* but rather were membrane associated. In contrast, Chang (29) reported that hemolytic factors were liberated from the amoebae. A heat-stable cytolytic protein associated with the surface membrane of *N. fowleri* and a heat-labile cytolysin from lysosome-enriched fractions have been described (131). Lowrey and McLaughlin (131) suggested that these interacting cytolytic components were part of a multicomponent system responsible for *N. fowleri* cytopathogenic and hemolytic activity. Although phospholipases and other enzymes are produced by *N. fowleri*, there is as yet no proof that they are unique virulence factors in vivo.

A major consideration in determining the mechanism of

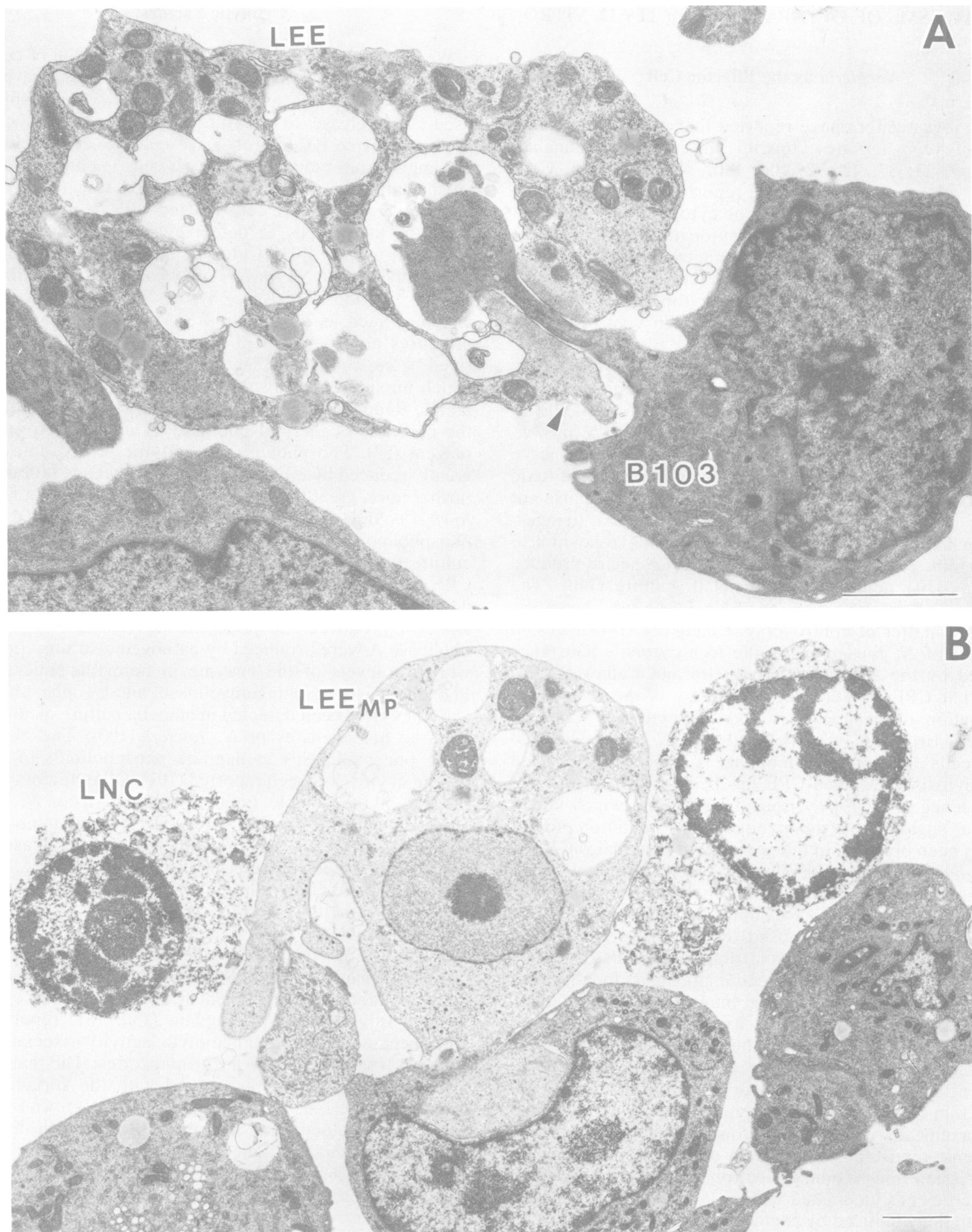

FIG. 3. Transmission electron micrographs of axenically grown and mouse-passaged *N. fowleri* amoebae cocultured with B103 rat neuroblastoma cells. (A) Ingestion of a portion of a B103 target nerve cell by axenically grown *N. fowleri* LEE after 18 h at 37°C. Food cups are used to damage the target cell (arrow). (B) Lysis of the B103 target cell by mouse-passaged *N. fowleri* LEEmp after 18 h at 37°C. LNC, Lysed nerve cell. Bar, 5 μm.

cell injury by *N. fowleri* is standardization of the conditions for assaying cytotoxicity since conflicting results concerning phagocytosis, trogocytosis, or the production of cytolytic factors have been obtained in different laboratories. The considerable differences which have been reported in terms of mechanisms of cell injury may be due to differences in strains of *Naegleria* used, target cells used, incubation and temperature conditions, or the growth cycle of the amoebae

at the time of assay. For example, amoebic lysates of *N. fowleri* LEE, NF-66, NF-69, and HB-4 are equally injurious to B103 nerve cells, whereas lysates of strains 6088 and KUL are less cytotoxic (81). Comparisons among strains and species are difficult because the conditions for cytopathic activity and the mechanism of cytotoxicity may differ. Earlier studies relied upon visual scoring for evaluation of cytopathogenicity (27, 37, 42). More recent experiments have used the ^{51}Cr release assay, which is a more sensitive and quantitative means of evaluating cytopathogenicity (81, 140). Various investigators have noted changes in pathogenicity of organisms maintained in vitro. Wong et al. (238, 239) noted that, after prolonged periods of maintenance in axenic medium, strains of *N. fowleri* lost their original pathogenicity for mice. In addition, differences in enzyme activity and in the ability to induce CPE in cell culture were noted in both highly and weakly pathogenic substrains. We have noted that axenically grown *N. fowleri* LEE and a serially mouse-passaged strain, LEEmp, differ considerably in the mechanism of cytopathogenicity. Although live amoebae and cell-free lysates of axenically grown LEE and LEEmp are cytopathogenic, axenically grown amoebae destroy nerve cells in culture by repeated nibbling or trogocytosis, while LEEmp destroys nerve cells by contact-dependent lysis (Fig. 3). Furthermore, the abundant food cups on axenically grown LEE which are used to ingest portions of target nerve cells are infrequently found on LEEmp (Fig. 1A and B).

Definition of the mechanism of *Naegleria* cytotoxicity is difficult since the length of time that amoebae are maintained in axenic culture following removal from brain tissue may affect expression of cytolytic substances. Furthermore, amoebae taken from brain tissue may contain host phospholipases or other host proteins which may affect target cell lysis in vitro. Finally, increased enzyme activities may be the result of host enzymes rather than those of amoebae.

Naegleria as a Target of Macrophages and Neutrophils

In addition to the interaction of *Naegleria* amoebae with continuous cell lines, the interaction of *N. fowleri* with primary cell cultures has been investigated. Murine resident peritoneal cells or peritoneal cells elicited with thioglycolate are destroyed by axenically cultured *N. fowleri* amoebae in a time, temperature, and multiplicity-dependent process by piecemeal ingestion of the cells via food cups (Fig. 1E). In contrast, murine macrophages activated in vivo by pyran, *Propionibacterium acnes* (*Corynebacterium parvum*) or Bacillus Calmette-Guerin (BCG) destroy *N. fowleri* amoebae in vitro under defined experimental conditions (31, 32, 136). The ability of macrophages to effect lysis of *N. fowleri* has been assessed by coincubating adherent peritoneal cells from $B_6C_3F_1$ mice with [^{3}H]uridine-labeled amoebae. Cell-cell contact was found to be requisite for cytolysis of amoebae by activated macrophages (Fig. 1F). Amoebicidal activity of activated murine macrophages was shown to be enhanced by *Naegleria*-specific antibody. In addition, conditioned medium from activated macrophages stimulated with *E. coli* lipopolysaccharide in vitro was capable of mediating cytolysis of *N. fowleri* amoebae. Resident macrophages, treated with lymphokine preparations from T-cell hybridomas for 24 h prior to addition of *N. fowleri* to the macrophage cultures, were capable of mediating a cytolytic response against the amoebae.

Macrophage cytolytic activity can be divided into two general categories: (i) that mediated by oxygen-dependent mechanisms and (ii) that mediated by oxygen-independent mechanisms. Oxygen-dependent mechanisms include the action of toxic oxygen intermediates produced by macrophages including hydrogen peroxide, superoxide anion, hydroxyl radical, and singlet oxygen. Oxygen-independent killing, on the other hand, is attributed to a number of soluble secreted factors including cytolytic protease, macrophage cytotoxin, or tumor necrosis factor. Results of studies from this laboratory indicate that the amoebicidal activity of activated macrophages is predominantly nonoxidative, since inhibitors of toxic oxygen metabolites and anaerobic conditions have been shown not to affect macrophage killing of *N. fowleri*. Rather, amoebicidal activity of activated macrophages appears to be the result of lipopolysaccharide-inducible soluble factors (32). In addition to activated macrophages, human neutrophils have been shown to kill *N. fowleri* in vitro only after exposure to lymphokine-containing supernatant fluids (78). However, the presence of specific antibody or complement was required to effect this killing. Mouse peritoneal neutrophils elicited with thioglycolate in vivo did not destroy *N. fowleri*. Peritoneal exudates rich in neutrophils, from mice injected intraperitoneally with live *N. fowleri*, destroyed *N. fowleri* amoebae. These authors suggested that lymphokines produced in vivo may have activated the neutrophils (75).

It appears that primary cell cultures are as susceptible to the cytopathic activity of *N. fowleri* amoebae as are continuous cell lines. However, cells of the immune system used as primary cell cultures, such as macrophages or neutrophils which have been activated by immunomodulators or lymphokines, are more resistant to the cytopathic action of *N. fowleri* amoebae.

HOST-PARASITE INTERACTIONS

Protective Immunity in Experimental Animals

Several animal models, including mice, monkeys, guinea pigs, sheep, and rabbits, have been used for studying PAME (18, 39, 61, 62, 95, 96, 148, 149, 170, 179, 198, 204, 220, 238). The mouse model has been used extensively to study the role of host immunological mechanisms in resistance or susceptibility to *N. fowleri* (96, 179). Mice infected intranasally develop a fatal disease resembling PAME in humans (18, 148). Mice immunized by repeated intravenous or intraperitoneal administration of sublethal doses of live amoebae develop species-specific agglutinating antibodies (97). Such antibodies have been produced also in mice immunized with formalinized and freeze-thawed amoebae (97, 215, 216). To date, all immunization regimens have produced modest protection against lethal infection, although a marked humoral response has been observed. More recently, Reilly et al. (179) reported that the bulk of the elevated immunoglobulins detected after stimulation with naeglerial antigens was not due to *Naegleria*-specific agglutinins. These authors noted elicitation of a polyclonal B-lymphocyte response in addition to the modest specific antibody response to *N. fowleri*. Although elevated levels of immunoglobulin M (IgM) and IgG were detected after immunization, only a small fraction of either immunoglobulin type could be absorbed with intact amoebae. Little or no IgM was absorbed by intact amoebae, while only 26% of total IgG1 was found to absorb. These investigators reported that most of the IgG1 was directed to unrelated antigens as a result of polyclonal activation of immunoglobulin production. Tew et al. (211) have observed that most specific antibody to naeglerial

antigens found in normal human sera is directed toward internal antigens rather than to surface antigens. The capability of lysates of *N. fowleri* to elicit polyclonal IgM activation has been assessed by using plaque formation, with sheep erythrocytes as the indicator. *N. fowleri* lysates, the 100,000 × *g* supernatant fluid, and the 100,000 × *g* sediment were found to enhance plaque-forming capability, indicating polyclonal B-cell activation by naeglerial antigens (179).

Conflicting results concerning the role of polyclonal B-cell activation have been presented (76, 179). Ferrante and Smyth (76) concluded that whole-amoeba antigen did not induce a polyclonal B-cell response. Rather, they suggested the presence of mitogenic activity for T suppressor cells in whole amoebic extracts prepared by freezing-thawing or by sonication. These investigators speculated that whole amoebic extracts did not elicit protective immunity because they contain mitogenic activity which enhanced T suppressor cells. In contrast, culture supernatant fluid from amoeba cultures used as an immunogen does not possess mitogenic activity for T suppressor cells and purportedly induces protection in mice challenged with lethal doses of *N. fowleri* (76, 215). Immunization with multiple doses of whole culture supernatant fluid harvested from *N. fowleri* cultures resulted in 78% protection against lethal *Naegleria* infection in mice (215). Concentrated *N. fowleri* culture supernatant fluids, fractionated on Sephacryl S200, yielded six peaks (fractions 1 to 6) which were used for immunization. A 100% survival rate occurred in mice immunized with fraction 1 containing protective antigens of >200,000 molecular weight. These results suggested that fraction 1 of the whole culture supernatant fluid contains less nonprotective antigens which can interfere with the production of protective antibody and that antigenic competition plays a role in the immune response to *N. fowleri* (215). The immunological mechanism responsible for the observed protection following immunization with culture medium from amoeba cultures was investigated by histological examination of the nasopharynx and brain (212). A marked inflammatory response was observed in immunized mice at the nasal mucosa, indicating protection at that site. Thus, a functional role for antibody in protective immunity was attributed to immobilizing the amoebae at the nasal mucosa, allowing for polymorphonuclear leucocytes to accumulate in the mucosa. Neutrophil-mediated killing of *N. fowleri* in vitro also has been demonstrated (75, 78).

Several studies have been undertaken to determine the effect of immunization of mice with *N. fowleri* on subsequent challenge with lethal doses of *N. fowleri*. Thong et al. (216) concluded that mice immunized intraperitoneally with live amoebae were more resistant to subsequent intranasal challenge. These authors suggested that antibody played an important role in immunity to *Naegleria* infections because resistance to *N. fowleri* could be passively transferred with serum from immunized animals but not by spleen cells (216). However, in many of these early studies, antibody titers to *N. fowleri* were not measured. Furthermore, while the majority of animals showed a delay in survival time, many eventually died from *Naegleria* meningoencephalitis (214, 216). In this context, Reilly et al. (179) were unable to transfer protective immunity to mice with immune serum, immune cells, or a combination of both. These authors suggested, therefore, that the humoral immune response in *Naegleria* infections was not correlated with protective immunity. In addition, *N. fowleri* can remove antibody from its surface by capping and internalizing surface-bound antibody (77). Failure to impair host resistance with cyclophosphamide or ^{60}Co radiation corroborated the proposition that humoral immunity is not a major line of host defense against PAME (179).

The inability of the host to develop strong protective immunity to *N. fowleri* suggests that either functional antigens which stimulate protective antibodies have yet to be identified or innate resistance plays a more important role than acquired immunity. Factors such as age and sex may affect susceptibility to *Naegleria* infection. Age has been shown to play a role in host susceptibility to *N. fowleri* in monkeys (238) and mice (95). Young animals were shown to be more susceptible to infection than adult animals. In addition, female mice were more resistant to infection than male mice (96).

Susceptibility to *N. fowleri* varies greatly among mouse strains. The most sensitive mouse is the complement-deficient (C5) strain (A/HeCr) (95). This observation indicates that innate resistance to *N. fowleri* may be provided by the complement system. Furthermore, complement depletion with cobra venom factor renders mice more susceptible to *N. fowleri* infection (179). Lysis of *N. fowleri* by human serum has been shown to be due to activation of complement, and the alternative complement pathway may be directly activated by this protozoan (99, 180). We have investigated the ability of human and guinea pig sera to lyse pathogenic and nonpathogenic *Naegleria* species in vitro to correlate resistance to complement-mediated lysis with pathogenic potential. The nonpathogenic species of *Naegleria*, *N. gruberi* and *N. lovaniensis*, and the weakly pathogenic species, *N. australiensis*, are more susceptible to lysis by normal human and guinea pig sera than the pathogenic species *N. fowleri* in vitro. In general, within the species *N. fowleri*, the more pathogenic the strain, the more resistant are the amoebae to complement-mediated lysis (232). These data indicate that complement is an important factor in host defense against PAME.

CMI

The role of cell-mediated immunity (CMI) to *Naegleria* spp. has been examined in guinea pigs (47, 62) and mice (179). Guinea pigs sensitized with *N. fowleri*, *N. gruberi*, or *N. jadini* developed delayed-type hypersensitivity when tested by intradermal injection with homologous or heterologous antigens of *Naegleria* spp. Delayed-type hypersensitivity was detected in 24 h by measuring the diameter of hard nodular lesions (62). CMI also was assessed by macrophage migration. When soluble freeze-thawed extracts of *N. fowleri*, *N. gruberi*, or *N. jadini* were added to peripheral blood lymphocytes of guinea pigs immunized with naeglerial antigens and cultured with macrophages, macrophage migration was inhibited (47). These results indicated that CMI was important in protection against *N. fowleri*. However, diethylstilbestrol, which depresses delayed-type hypersensitivity, did not alter host resistance to *N. fowleri* in $B_6C_3F_1$ mice (179). Furthermore, mortality due to *N. fowleri* in congenitally athymic mice, which are deficient in T-cell activities, did not differ from that in euthymic mice (157). These results indicate that the role of CMI in resistance to *Naegleria* infection awaits further definition.

Antibodies in Human Sera

Serological surveys of apparently healthy humans have been conducted to determine the presence of antibodies to *Naegleria* spp. (46, 139, 178). In one study, antibodies were found in all 200 sera tested from three New Zealand health

districts. Indirect immunofluorescence assays indicated the presence of antibodies for pathogenic and nonpathogenic *Naegleria* spp. with titers ranging from 1:5 to 1:20 (46). Sera from 423 human subjects from Virginia and North Carolina were assessed for agglutinating antibodies to *N. fowleri* and *N. gruberi*. Sera from the umbilical cords of seven infants failed to agglutinate *N. fowleri* or *N. gruberi* (178). However, results of studies on antibody titers to *Naegleria* spp. in cord blood samples performed in our laboratory differ from those of Cursons et al. (46), who reported that cord serum contained antibodies to *N. fowleri* and *N. gruberi*. Differences in the agglutination and immunofluorescence assays, parameters used in the two laboratories, may account for the discrepancy. Since agglutination assays detect IgM antibodies which are not passed transplacentally to newborns, the presence of maternal IgG antibodies in cord sera could account for the antibodies detected by the immunofluorescence assay in the study of Cursons et al. (46).

There are accumulating data which indicate that humans are periodically exposed to *Naegleria* spp. (139). Studies performed with sera of adolescents and young adults have shown the presence of anti-*Naegleria* antibodies with a median agglutination titer of 1:16, suggesting early exposure to immunogen in sufficient amounts to elicit a measurable immune response. The agglutinating activities were species specific, suggesting that the agglutinating activity for *N. fowleri* is not the result of prior exposure to *Naegleria* heterospecies (e.g., *N. gruberi*). The agglutinating antibody was of the IgM class, suggesting host exposure to low concentrations of naeglerial antigen, on the one hand, or that immune responsiveness was elicited to *Naegleria* components such as repeating polymers, e.g., polysaccharides or glycolipids (178). Serum obtained periodically from a patient with PAME before death showed no elevation in specific antibody titer by indirect immunofluorescence. Radioimmunodiffusion for quantitating serum IgM, IgG, and IgA revealed a low level of serum IgA (48). In a fatal case of PAME from England, patient immunoglobulin levels on day 3 of clinical disease were within normal limits (14). In a patient who survived PAME in the United States, specific anti-*Naegleria* antibodies were identified in serum by immunofluorescence up to a dilution of 1:4,096 at 7, 10, and 42 days after hospital admission (195). However, serological data to date have not allowed for correlation of susceptibility to PAME and the immune status of the afflicted human subject.

Parasite Factors

The capability of human serum samples to agglutinate rounded cells but not live amoebae of *N. fowleri* and *N. gruberi* is consistent with the hypothesis that rounded cells display unique antigenic sites on their surfaces (178). Indeed, amoebae of *N. fowleri* may redistribute or internalize their surface antigens, affording a mechanism to avoid deleterious effects of specific antibody. In addition, *Naegleria* amoebae may become coated with immunoglobulin molecules, thereby presenting "self" surface antigens to the host, thus providing another mechanism for evading humoral host defenses. *Naegleria* amoebae may extrude internal antigens or shed surface antigens which may, on occasions, deplete amoebicidal antibodies. The resultant circulating antigen-antibody complexes may deplete serum complement. Hypergammaglobulinemia, whether resulting from polyclonal activation of lymphocytes or misdirected antibody response to "nonprotective" antigens of *Naegleria*, may subvert the immune response with the consequence that protective antibodies are not elicited.

Carbohydrates are major constituents of the outer surface of *Naegleria* plasma membranes. However, sugars manifested on the surface of *Naegleria* species are different. Concanavalin A agglutinates *N. gruberi* (207) and *N. lovaniensis* (205), but not *N. jadini* or *N. fowleri* (108). Concanavalin A, in the presence of mannose, does not agglutinate the nonpathogenic species *N. lovaniensis* (205). Strains of *N. australiensis* differ in their capacity to agglutinate with concanavalin A, with nonagglutinating strains apparently being the most virulent (56). Wheat germ agglutinin agglutinates *N. fowleri* and *N. gruberi*. The wheat germ agglutinin-induced agglutination of both species is reversed by competition with *N*-acetylglucosamine (207). In contrast, *N. lovaniensis* is not agglutinated by wheat germ agglutinin (205). Species and strains of pathogenic and nonpathogenic *Naegleria* cannot be separated on the basis of lectin agglutinability (196).

Several investigators have suggested that *N. fowleri* releases cytolytic substances which account for invasiveness and tissue damage in vivo and for cytopathogenicity in vitro (27, 29, 37, 220). Indeed, electron microscopy of brain from mice infected with *N. fowleri* revealed areas of extensive demyelinization containing trophozoites surrounded by a clear "halo" (18, 149, 220). It has been proposed that the release of phospholipolytic enzymes by *N. fowleri* results in the rapid destruction of brain tissue (29). Acid phosphatase activity of *N. fowleri* amoebae in CNS lesions has been detected (74). Acid phosphatase has been detected also, in vivo along membranes of the host-parasite interface. Feldman (74) suggested that membranous fronds with hydrolytic activity were released from the amoeba plasmalemma. Fronds in contact with host cells, then, initiated hydrolytic activity. However, *N. fowleri* phagocytic attack of host cells by pseudopodial extensions also has been observed (220). Visvesvara and Callaway (220) have suggested that a cytolytic material is released during, or prior to, this phagocytic process.

The ability of the amoebae to attach to and penetrate the nasal mucosa or to exhibit an increased rate of locomotion or both may be determinative parameters in production of disease. Another important aspect of *Naegleria* pathogenesis is the ability of pathogenic amoebae to evade the host immune system. To date, the factors which determine susceptibility to *Naegleria* infection and subsequent development of PAME have not been defined. There is no evidence for hereditary immunologic deficiencies. It is conceivable that transitory complement depletion would increase susceptibility to a challenge with *N. fowleri*. Complement deficiency could be elicited by a gram-negative infection (enteric food poisoning or exposure to an environmental chemical such as dioxin). It is well established that recent animal passage enhances pathogenicity. It is conceivable that amoebae released from an infected animal may be the effective etiologic agent of PAME. Pathogenicity, of course, is related to challenge level, so exposure to exceptionally high numbers of *N. fowleri* may be the determinative factor. The ability to survive and grow at temperatures of 37°C and above does not appear to determine pathogenicity since thermophilic nonpathogenic species such as *N. lovaniensis* have been described. Likely determinative factors however, may be the following: (i) recent animal passage, (ii) resistance to complement-mediated lysis, (iii) chemokinesis elicited by mammalian cells and tissue fluids, and (iv) evasion of host immunity.

CONCLUDING REMARKS

The genus *Naegleria* is composed of a distinctive group of free-living amoebae which are widespread in nature. It is not clear whether there are animal or plant reservoirs of *Naegleria* species. Only two attributes of members of this genus have been studied in detail, enflagellation in *N. gruberi* (64, 84) and pathogenicity of *N. fowleri* (18, 148). Classification of these organisms has relied largely on morphological characteristics. *Naegleria* species can be readily cultivated axenically, but chemically defined media do not support the same rate and extent of growth as complex media containing serum, yeast extract, liver digest, or proteose peptone. *Naegleria* spp. are typical eucaryotic protozoan cells with intricate microfilamentous arrays. *Naegleria* species are particularly useful tools for studying gene expression during differentiation. Both *N. fowleri* (166, 240–242) and *N. gruberi* (64, 83, 84) enflagellation have been examined in detail; however, encystment cannot be manipulated experimentally as successfully as enflagellation. *Naegleria* species respond chemokinetically and chemotactically to a number of exogenous signals such as bacteria, eucaryotic cells, and cell products (33, 138). Although a number of enzymes have been detected in *Naegleria* spp., few of these have been isolated and characterized. Furthermore, few metabolic pathways and control mechanisms have been identified. The mechanisms by which *Naegleria* species injure, kill, and digest target food sources have not been resolved. Bacterium-sized particles are ingested, sometimes by a unique food cup. Large target cells may be ingested, injured by nibbling (trogocytosis), or lysed. All *Naegleria* species examined to date can kill and live upon mammalian cells in culture, once appropriate conditions are used. *N. gruberi*, for example, grows poorly at 37°C and does not display appreciable cytopathogenicity for mammalian cells in culture at 37°C unless a high multiplicity of infection is used (137, 140, 143). *N. fowleri* presents an interesting host-parasite interaction in which complement activity is an important host defense mechanism. *N. fowleri* and mammalian macrophages can be manipulated to reverse their target/effector roles (31, 32, 136). This constitutes one of the more intriguing models for the study of cell-cell interactions.

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