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Acanthamoeba spp. as Agents of Disease in Humans

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INTRODUCTION

Free-living amebae belonging to the genus *Acanthamoeba* are the causative agents of granulomatous amebic encephalitis (GAE), a fatal disease of the central nervous system (CNS), and amebic keratitis (AK), a painful sight-threatening disease of the eyes (95, 210, 286, 325). *Acanthamoeba* spp. also have been associated with cutaneous lesions and sinusitis in AIDS patients and other immunocompromised individuals (128, 143, 164, 179, 282, 295, 446). The first suggestion that *Acanthamoeba* could cause disease in humans came in 1958 during polio vaccine safety trials. Plaques appeared in cell cultures used to prepare vaccine and were thought to be virus induced because mice and monkeys died from encephalitis following inoculation of tissue culture fluid. However, these plaques were found later to be caused by amebae (98, 99). Both trophozoites and cysts were detected in cell cultures and were identified as belonging to the genus *Acanthamoeba.* These observations of experimental animals dying from encephalitis led Culbertson et al. (99) to predict a role for free-living amebae as agents of human disease. Human cases of amebic encephalitis were reported soon thereafter from Australia, Europe, Africa, South America, and the United States (35, 57, 58, 64, 74, 142, 201, 280, 284, 344, 476). However, some of these cases were identified later as primary amebic meningoencephalitis, a rapidly fatal disease of the CNS caused by another free-living ameba, *Naegleria fowleri* (57, 268, 286). The first cases which clearly established *Acanthamoeba* as causative agents of disease in humans were reported in the early 1970s. These included reports of amebic encephalitis, amebic keratitis, and skin infections (164, 201, 210, 213, 284, 325, 368, 374, 476). Consequently, since different free-living amebae can infect the CNS, the term "granulomatous amebic encephalitis" (GAE) has been used for CNS infections caused by *Acanthamoeba* spp. while the term "primary amebic meningoencephalitis" has been reserved for CNS infections caused by *Naegleria fowleri*

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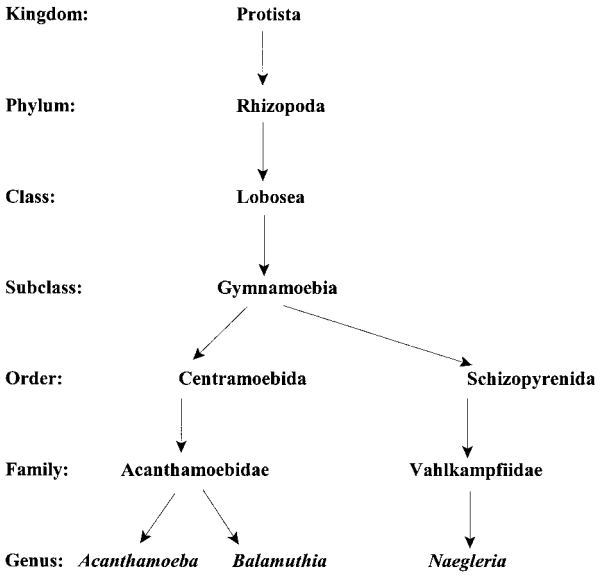

FIG. 1. Phylogenetic scheme of *Acanthamoeba*, *Balamuthia*, and *Naegleria.* Modified from references 91 and 378.

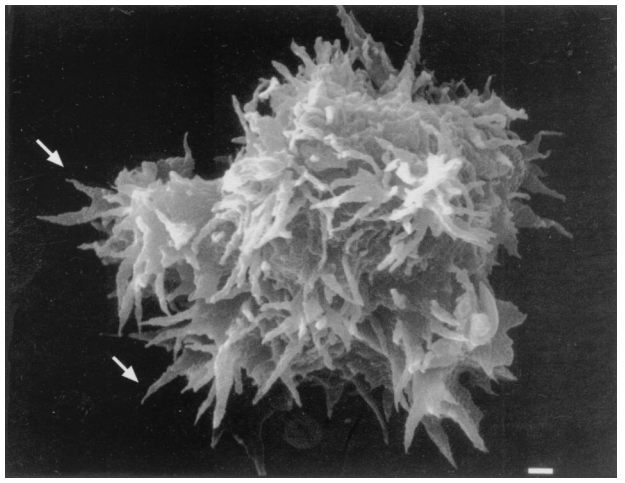

FIG. 2. Scanning electron micrograph of an *Acanthamoeba* trophozoite. Spiny surface structures called acanthopodia (arrows) distinguish *Acanthamoeba* from other free-living amebae that infect humans, such as *B. mandrillaris*, *N. fowleri*, and *Sappinia diploidea.* Bar, 1 μm.

(64, 286). *Acanthamoeba* and *Naegleria* have been termed amphizoic organisms since they have the ability to exist both as free-living amebae and as parasitic pathogens (341). More recently, two other free-living amebae from distinct genera, *Balamuthia mandrillaris* and *Sappinia diploidea*, have been associated with CNS infections in humans (156, 461). *B. mandrillaris* was reported to cause fatal amebic encephalitis in both healthy and immunosuppressed patients (113, 281, 387). *S. diploidea*, a soil ameba, was identified in an otherwise healthy individual who experienced nonfatal amebic encephalitis following a sinus infection (156). Thus, it is becoming increasingly apparent that free-living amebae cause human disease. Furthermore, with increasing awareness of the potential of free-living amebae to cause disease, amebae from other genera may be found to be causative agents of human infections.

CLASSIFICATION OF *ACANTHAMOEBA*

Acanthamoeba was first described by Castellani when he reported the presence of an ameba in *Cryptococcus pararoseus* cultures (70). The genus *Acanthamoeba* was established later by Volkonsky in 1931 (463), but the actual classification of organisms within this genus is currently under review (12, 41, 42, 50, 56, 61, 151, 232, 395, 434). *Acanthamoeba* has been placed in the Family Acanthamoebidae (Fig. 1). A second genus, *Balamuthia*, previously assigned with amebae of uncertain affinities, has recently been included in this family (91, 378). Studies suggested that the genus *Balamuthia* be transferred from the family Leptomyxidae to Acanthamoebidae on the basis of molecular analysis of 16S-like rRNA genes (12, 434). Furthermore, *Acanthamoeba* and *Balamuthia* both possess a multilayered microtubule-organizing center and both can cause disease in humans (345). Identification of *Acanthamoeba* at the genus level is relatively easy due to the presence of spiny surface projections, termed acanthopodia, on trophozoites (Fig. 2). However, using morphological criteria, identification of these amebae at the species level has been difficult. *Acanthamoeba* spp. have been placed into three morphological groups (I, II, and III) based on cyst size and shape (340, 363). Species in group I were designated on the basis of having a large cyst in comparison to that of species in the other groups. Species in group II were characterized as having a wrinkled ectocyst and an endocyst which could be stellate, polygonal, triangular, or oval. Species in group III typically exhibited a thin, smooth ectocyst and a round endocyst. Nevertheless, classification of *Acanthamoeba* based on morphological characteristics of the cyst wall has proved unreliable because cyst morphology can change depending on culture conditions (15, 105, 390, 435). Immunological, biochemical, and physiological criteria also have been applied to the identification of different species of *Acanthamoeba* (10, 92, 189, 219, 462, 466, 467). However, many species share antigenic determinants. Therefore, results obtained through immunological approaches such as Western blotting and immunofluorescence have been inconclusive in identifying species. Isoenzyme electrophoresis of different enzyme systems also has been used to compare strains of *Acanthamoeba* (105, 112). Although this method has the potential to provide insight into relationships among species, results have indicated interstrain variation within species as well as similarities between strains of separate species. Furthermore, studies have shown that enzyme patterns change when isolates are grown under different laboratory conditions (199, 472).

To address these potential confounds, methods for classification of *Acanthamoeba* species at the molecular level have been developed (10, 39, 42, 62, 83, 152, 209, 225, 231, 395). A number of laboratories have applied mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis to cluster strains of *Acanthamoeba* (39, 154, 219, 483). However, Gast et al. (152) reported that although assessment of mitochondrial DNA was useful for typing *Acanthamoeba* isolates, an inherent drawback to this approach was the relatively large number of amebae required for analysis. Johnson et al. (209)

TABLE 1. *Acanthamoeba* spp. isolated from the environment and from humans

Species[a]	Type strain[b]	Sequence type[c]	Group[d]	Isolation[e]	Reference(s)
A. astronyxis	30137	T7	I	Water from termite colony	340, 366
A. castellanii	30011	T4	II	Yeast culture	70, 119, 463
A. commandoni	30135	T9	I	Garden humus	90, 362
A. culbertsoni	30171	T10	III	Monkey kidney cell culture	99, 413
A. divionensis[i]	50238	—[f]	II	Soil	363
A. echinulata	50239	—	I	Compost	363
A. griffini	30731	T3	II	Seawater bottom sample	389
A. hatchetti	30730	T11	II	Harbor sediment	393
A. healyi	CDC:1283:V013[g]	T12	III	Brain tissue	320
A. jacobsi	30732	—	III	Marine sediment	392
A. lenticulata	30841	T5	III	Swimming pool	313
A. lugdunensis	50240	T4	II	Pool	350, 363
A. mauritaniensis	50253	T4	II	Sewer sludge	363
A. palestinensis	30870	T2	III	Soil	340, 367
A. pearcei	50435	T3	I	Sewage sediments	327
A. polyphaga	CCAP1501/3A[h]	T4	II	Pond	340, 361
A. pustulosa[j]	50252 (GE3a)	T2	III	Pool	112, 350, 363
A. quina	50241	—	II	Swimming pool	350, 363
A. rhysodes	30973	T4	II	Soil	418
A. royreba	30884	T4	III	Human choriocarcinoma cells	478
A. stevensoni	50438	T11	II	Shellfish beds	391
A. triangularis	50254	T4	II	Human feces	363
A. tubiashi	30867	T8	I	River water	259

[a] Identification of strains should be based on comparisons with type strains (395).
[b] ATCC designation.
[c] Genotypes Rns T1 to T12 based on Ohio State University *Acanthamoeba* nuclear small-subunit ribosomal DNA (rDNA). Available at www.biosci-ohiostate/~tbyers/byers.htm.
[d] Genera are divided into three morphological groups based on cyst size and shape (363).
[e] Original isolation of type strain.
[f] —, sequence type not yet determined (395).
[g] CDC, Centers for Disease Control and Prevention.
[h] CCAP, Culture Collection of Algae and Protozoa.
[i] *A. paradivonensis* may be the same as *A. divionensis.*
[j] *A. pustulosa* (*A. palestinensis*) 50252 GE3a.

used reverse transcription to determine the partial nucleotide sequences of small-subunit rRNAs of ameba isolates. They reported a high degree of 18S rRNA sequence diversity within the genus *Acanthamoeba.* However, subsequent DNA sequencing results from a number of laboratories have not confirmed these observations, and so conclusions regarding large sequence differences among *Acanthamoeba* strains appear unwarranted. As opposed to the RNA sequencing approach reported by Johnson et al. (209), Gast et al. (152) developed a classification scheme based on nuclear rRNA gene sequences (18S rDNA). The complete gene sequence of nuclear small ribosomal subunit RNA (Rns) was determined. Using this approach, Stothard et al. (434) classified 53 isolates of *Acanthamoeba* species on the basis of 12 rDNA sequence types (Rns genotypes) designated typing units T1 to T12 (Table 1). Additional sequence types may exist (151). Sequences of either nuclear (Rns) or mitochondrial (rns) rRNA genes are suitable for classifying isolates. Current classification schemes integrate the morphological groups which were established by Pussard and Pons (363) with the 12 sequence types (Rns genotypes) (T1 to T12) such that group I includes sequence types T7, T8, and T9, group II includes sequence types T3, T4, and T11, and group III includes sequence types T1, T2, T5, T6, T10, and T12. Studies in which clinical isolates have been identified based on sequence types have shown that the majority of strains causing keratitis belong to sequence type 4 (i.e., T4) (395, 434, 464). Chung et al. (83) used a riboprinting approach for subgenus classification of *Acanthamoeba.* Genomic DNA was extracted, and small-subunit rDNA was amplified by PCR and digested with restriction enzymes for analysis of RFLP. The resultant dendrogram based on riboprinting coincided with the grouping scheme of Pussard and Pons (363), which was based on morphological criteria, and with that of Stothard et al. (434), who examined 18S rDNA gene sequence variation (Table 1). In summary, comparison of results for classification of *Acanthamoeba* species obtained through DNA-based approaches with those based on morphological and biochemical criteria has revealed major inconsistencies. A revision of the taxonomy of the genus based on sequence comparisons is under way (42, 61).

BIOLOGY AND DISTRIBUTION OF *ACANTHAMOEBA*

Ecology and Distribution

Acanthamoeba spp. are among the most prevalent protozoa found in the environment (301, 340, 369, 370, 376). They are distributed worldwide and have been isolated from soil, dust, air, natural and treated water, seawater, swimming pools, sewage, sediments, air-conditioning units, domestic tap water, drinking water treatment plants, bottled water, dental treatment units, hospitals and dialysis units, eyewash stations, and contact lenses and lens cases and as contaminants in bacterial, yeast, and mammalian cell cultures (23, 68, 70, 111, 202, 228,

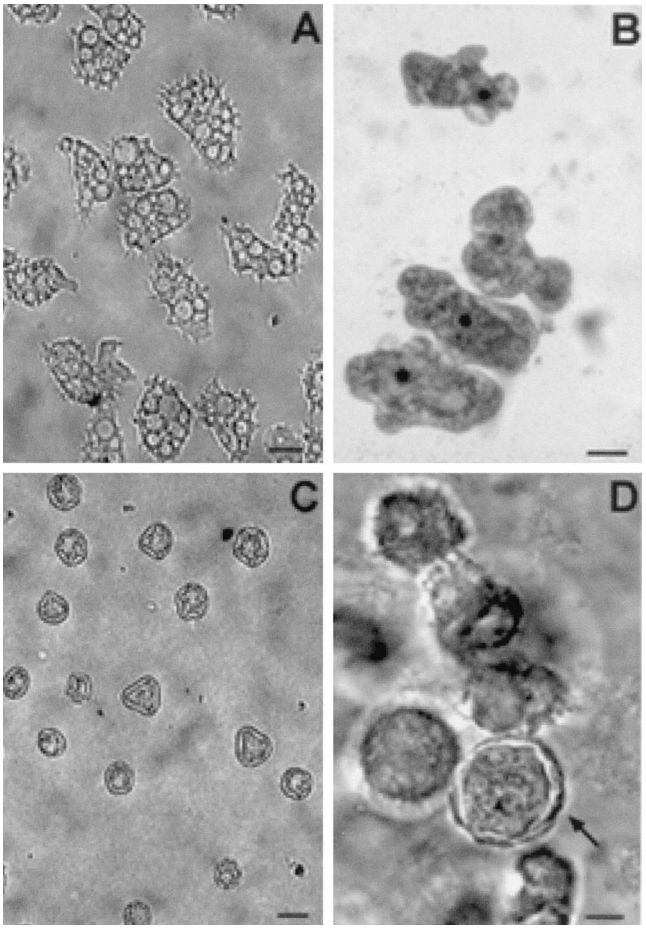

FIG. 3. Light micrographs of cultures depicting life cycle stages of *Acanthamoeba* spp. (A and C) Unstained preparations of cultures of *A. astronyxis* trophozoites (A) and cysts (C). (B and D) H-&-E-stained preparations of *A. castellanii* trophozoites (B) and cysts (D). Bars, represent 50 μm (A and C) and 25 μm (B and D).

301, 305, 343, 372, 412, 437). *Acanthamoeba* spp. also have been isolated from vegetation, from animals including fish, amphibia, reptiles, and mammals (129, 266, 267, 465), from the nasal mucosa and throats of apparently healthy humans (75, 304, 330), from infected brain and lung tissue, from skin lesions of immunosuppressed patients, and from corneal tissue of patients with AK (111, 245, 286).

Life Cycle

The life cycle of *Acanthamoeba* consists of two stages: an actively feeding, dividing trophozoite and a dormant cyst (Fig. 3 and 4). The trophozoite varies in size from 25 to 40 μm and feeds on bacteria, algae, and yeast in the environment but also can exist axenically on nutrients in liquid taken up through pinocytosis (44, 47). Uptake of food by trophozoites can occur by pseudopod formation and phagocytosis or by food cup formation (Fig. 5) and ingestion of particulate matter. Food cups formed on the ameba surface are temporary structures used to ingest bacteria, yeast, or cells (351). Locomotion involves the formation of a hyaline pseudopodium and is sluggish in all species of *Acanthamoeba* (358). One species, *A. castellanii*, has been used extensively to study the molecular mechanisms of actin polymerization during ameboid locomotion (235, 356).

Morphology

The cellular organization of *Acanthamoeba* has been studied using electron microscopy (45, 46, 158, 379). Organelles typically found in higher eucaryotic cells have been identified in *Acanthamoeba* (Fig. 4). Bowers and Korn (45) indicated the presence of a Golgi complex, smooth and rough endoplasmic reticula, free ribosomes, digestive vacuoles, mitochondria, and microtubules in *Acanthamoeba* trophozoites. A trilaminar plasma membrane was found to surround the cytoplasmic contents of the trophozoite. In addition, distinguishing features of the trophozoite were the presence of spiny surface projections called acanthopodia (Fig. 2), a prominent contractile vacuole in the cytoplasm that controls the water content of the cell, and a nucleus with a large central nucleolus. Generally, the amebae are uninucleate, although multinucleated cells are common when *Acanthamoeba* are maintained in suspension culture. Reproduction occurs by binary fission (59, 340).

A double-walled wrinkled cyst (Fig. 4) composed of an ectocyst and an endocyst ranges in size from 13 to 20 μm and varies from species to species (46). Cyst formation occurs under adverse environmental conditions such as food deprivation, desiccation, and changes in temperature and pH (46, 60, 76). Villemez and coworkers (457, 484) have reported that antibody binding to a specific membrane protein also causes *A. castellanii* to encyst. Cysts are resistant to biocides, chlorination, and antibiotics (109, 217, 262, 448) and survive low temperatures (0 to 2°C) (55). Meisler et al. (300), however, have shown that treatment with Freon or methylene oxide or autoclaving destroys cysts. Excystment occurs when trophozoites emerge from the cyst under suitable environmental conditions. Mazur et al. (297) demonstrated that cysts retained viable amebae for over 24 years after storage in water at 4°C. Of 17 environmental isolates of *A. polyphaga* or *A. castellanii* maintained as cysts, 14 gave rise to trophozoites on inoculation on nonnutrient agar (NNA) containing bacteria. After the excystment process, amebae were tested for pathogenicity by intranasal inoculation of BALB/c mice. Fewer deaths were recorded for mice inoculated with amebae which had been encysted for 24 years than for mice inoculated with the same environmental isolates when tested initially (297). Thus, although virulence was shown to decline with the passage of time, the isolates retained pathogenicity for mice.

Because *Acanthamoeba* trophozoites can be induced to transform into cysts in nonnutrient media and excystation occurs under favorable conditions, the amebae have been used to study differentiation. *Acanthamoeba* has been used as a model system to study eucaryotic RNA transcription, RNA polymerase functions, and cellular differentiation in trophozoites and cysts (29, 63, 79, 190, 203, 338, 396). Paule and coworkers (346) have purified RNA polymerases from *Acanthamoeba* and have performed extensive studies on initiation and regulation of RNA transcription in *Acanthamoeba* (8, 194, 347, 348, 355, 364).

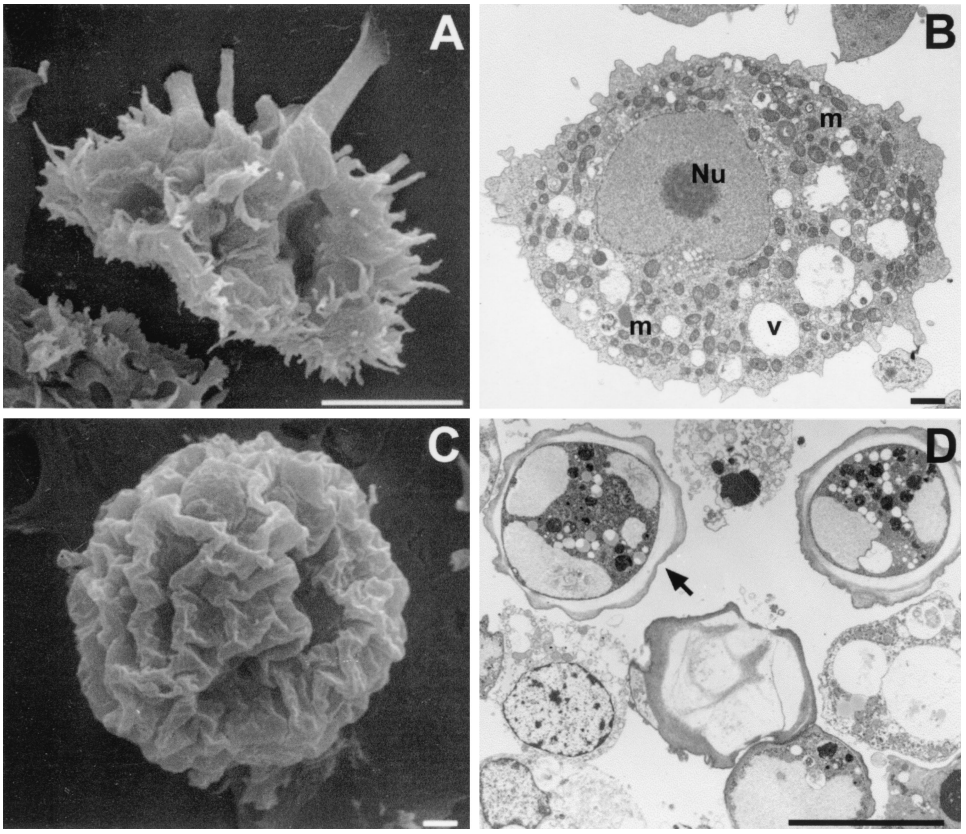

FIG. 4. Scanning (A and C) and transmission (B and D) electron micrographs depicting the life cycle stages of *Acanthamoeba spp.* (A) *A. polyphaga* trophozoite; (B) trophozoite of *A. castellanii* showing the prominent central nucleolus (Nu), mitochondria (m), and cytoplasmic food vacuoles (v); (C) wrinkled cyst of *A. polyphaga*; (D) double-walled cyst of *A. castellanii.* Bars, 10 μm (A and D) and 1 μm (B and C).

CULTURE METHODS FOR *ACANTHAMOEBA*

A variety of undefined liquid media, different mammalian cell types, and NNA seeded with bacteria support the growth of *Acanthamoeba* spp. *Acanthamoeba* can be grown on NNA (1.5%) containing a lawn of *Escherichia coli* (458). *Acanthamoeba* also can be grown axenically in PYG medium consisting of 2% proteose peptone, 0.2% yeast extract, and 0.1 M glucose (459) or in Oxoid medium (Cline medium) containing serum and hemin, which has been used to culture *Naegleria* spp. (268). Mammalian cells which support the growth of *Acanthamoeba* include African green monkey kidney (Vero), human embryonic lung (HEL), human embryonic kidney (HEK), HeLa, B103 rat neuroblastoma, and L929 fibroblasts (101, 110, 351). Conditions and medium formulations for the cultivation of pathogenic and opportunistic free-living amebae have been reviewed recently (399).

ACANTHAMOEBA SPP. AS OPPORTUNISTIC PATHOGENS

Granulomatous Amebic Encephalitis

GAE is a disease which is characterized by a chronic protracted slowly progressive CNS infection (Fig. 6) which also may involve the lungs (127, 284). Serological laboratory diagnosis has indicated that several species of *Acanthamoeba* are associated with GAE. Although the incubation period for *Acanthamoeba* infections is unknown, several weeks or months may be necessary to establish clinical signs. Table 2 summarizes differences in terms of disease, portal of entry, clinical signs, pathology, and diagnosis for free-living amebae causing human infections. GAE is generally associated with individuals who already have underlying diseases such as malignancies (133), systemic lupus erythematosus (163, 230), diabetes (172), renal failure, cirrhosis, tuberculosis, skin ulcers, human immunodeficiency virus (HIV) infection or Hodgkin's disease (201, 277, 286, 426, 477). Predisposing factors include alcoholism, drug abuse, steroid treatment, cancer chemotherapy, radiotherapy, and organ transplantation (14, 282, 408). Although enhanced susceptibility to infection is associated with immune suppression and debilitating conditions, cases of GAE caused by *Acanthamoeba* have been found in immunocompetent children and adults (35, 336, 368, 388, 419). The route of infection is thought to be by inhalation of amebae through the nasal passages and lungs or introduction through skin lesions. Access to the CNS may be by hematogenous spread from a primary site in the lungs or skin or directly through the olfactory neuroepithelium (282). Symptoms of CNS infection (Table 3) include headache, confusion, nausea, vomiting, fever, lethargy, stiff neck, focal neurologic deficits, or signs of increased intracranial pressure (280, 286). Pathological findings generally include severe hemorrhagic necrosis, fibrin thrombi, and inflam-

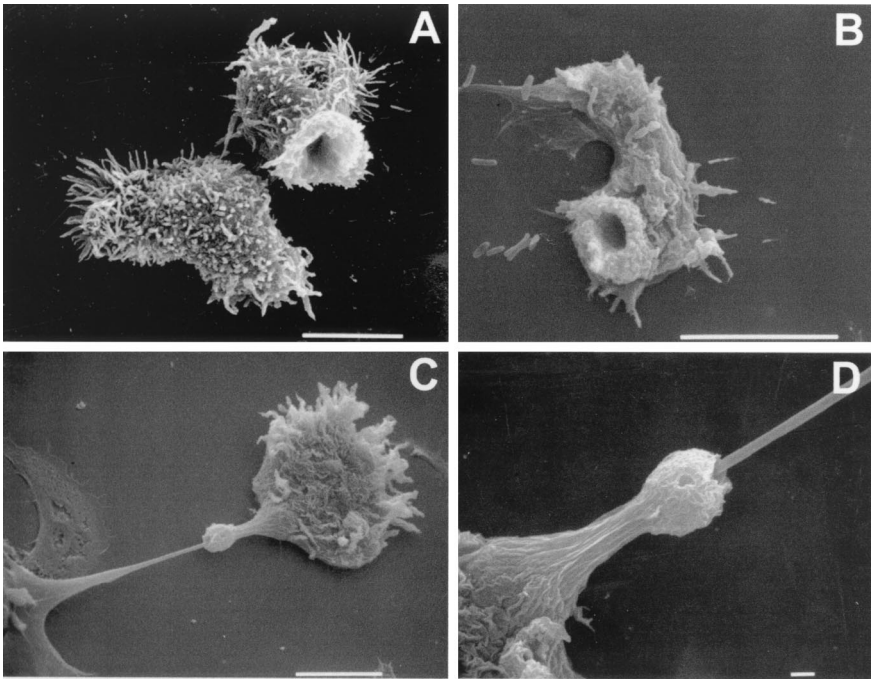

FIG. 5. Scanning electron micrographs of trophozoites illustrating the presence of surface structures termed food cups. (A) Food cups present on the surface of a trophozoite of *A. culbertsoni* are temporary structures that form and reform for the intake of bacteria, yeast, or cellular debris. (B) Food cup present on the surface of *A. astronyxis* trophozoite used to ingest bacteria. (C) Food cup present on the surface of an *A. castellanii* trophozoite in the apparent process of ingesting a cultured nerve cell. (D) Higher magnification of the trophozoite in panel C to illustrate the food cup structure in the apparent process of ingestion. Bars, 10 μm (A to C), and 1 μm (D).

mation. The cerebral hemispheres show moderate to severe edema. Multifocal lesions are present in the midbrain, brain stem, corpus callosum, and cerebellum. A chronic inflammatory exudate is observed over the cortex and is composed mainly of polymorphonuclear leucocytes and mononuclear cells. Severe angiitis with perivascular cuffing by lymphocytes is seen in some cases. In addition, numerous trophozoites can be identified within tissue (Fig. 7). However, other than these clinical and laboratory observations, there is a paucity of information concerning the pathogenesis of infection and the response of the host to infection, particularly as it involves the CNS. For example, in immunocompetent individuals, well developed granulomas form (Fig. 8) around the organisms, while in immunocompromised individuals, granuloma formation is weak or lacking (277) Furthermore, it is unknown whether severe necrosis of the brain is due to direct destruction of tissue by *Acanthamoeba* trophozoites or by induction of inflammatory cytokines such as interleukin-1 (IL-1) or tumor necrosis factor (TNF-α) or through the interactive action of both pathways (271). In addition, dissemination of amebae to other organs such as the liver, kidneys, trachea, and adrenals can occur in immunocompromised individuals (214, 277, 282, 322). Individuals with GAE also may have lung involvement. Trophozoites and cysts have been found in pulmonary alveoli from infected individuals, and pneumonitis is a characteristic feature (149, 178, 214, 278, 460). *Acanthamoeba* has also been recovered from ear infections (257) and from necrotic bone tissue of a patient with osteomyelitis of a bone graft of the mandible (43).

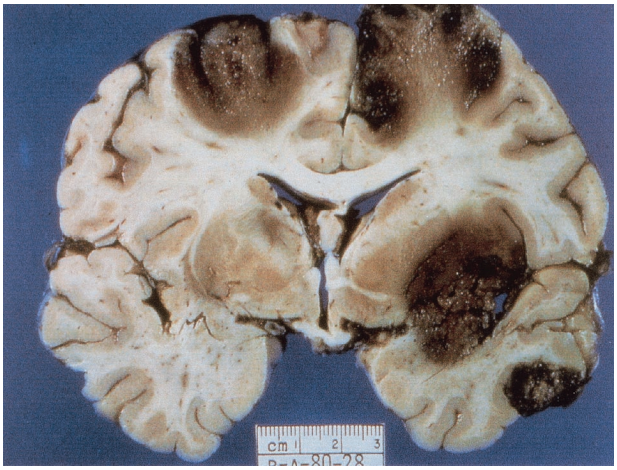

FIG. 6. Coronal section of the cerebral hemispheres with cortical and subcortical necrosis from a fatal human case of GAE. (Courtesy of A. J. Martinez; reprinted from reference 277 with permission of the publisher.)

Infections in Patients with AIDS

The first reported case of *Acanthamoeba* infection in a patient with AIDS was in 1986 (157). Since then, an increasing number of cases of disseminated *Acanthamoeba* infections have been reported in individuals with AIDS (40, 65, 69, 77, 125, 128, 143, 149, 159, 173, 178, 192, 214, 224, 258, 295, 307, 322, 373, 375, 380, 406, 407, 420, 438, 442, 446, 474) (see Table

4). Most of these have been diagnosed postmortem (65, 143, 322, 407, 420, 474). It has been postulated that impairment of host defense mechanisms in immunocompromised individuals results in, or contributes to, infection which can spread from the primary site of infection to other organs and tissues. In immunosuppressed individuals, a well-developed granulomatous reaction may not occur. Such individuals usually exhibit advanced HIV disease with a low $CD4^+$ T cell count (less than 200/mm^3) at the time of infection with *Acanthamoeba* (173). The clinical course can be fulminant, with rapid progression to death. Most patients die in less than 1 month after onset of neurological symptoms (65, 149, 159, 406, 438, 474). While cerebrospinal fluid (CSF) lymphocytosis is observed in non-AIDS *Acanthamoeba*-infected individuals, CSF may be devoid of cells in HIV-positive patients (149). Other prevalent manifestations of *Acanthamoeba* infections in HIV-positive individuals are chronic sinusitis, otitis (115, 224, 373, 442), and cutaneous lesions with *Acanthamoeba* organisms present in sinus lesions and skin ulcers (40, 69, 128, 157, 224, 442, 446). The nasal passage is thought to be the portal of entry for *Acanthamoeba*, although skin lesions may serve as the primary site of infection. Indeed, skin lesions are most often the presenting manifestation of *Acanthamoeba* infection in AIDS patients (322, 438). Also, there have been reports of separate cases of leukocytoclastic vasculitis, amebic osteomyelitis, and endophthalmitis in AIDS patients with *Acanthamoeba* infections (178, 179, 407). Because other opportunistic infections occur in AIDS patients, those due to *Acanthamoeba* are often overlooked. In fact, patients with CNS symptoms have been diagnosed empirically with toxoplasmosis, although serological testing for *Toxoplasma* may be negative (149, 159, 173, 406). Other patients have been misdiagnosed with CNS vasculitis (159), squamous cell carcinoma (373), or bacterial meningitis (173). *Acanthamoeba* in tissues also has been identified incorrectly as macrophages or fungi (69, 157, 214, 258, 271, 322, 337, 420, 438, 474).

Cutaneous Acanthamebiasis

Cutaneous infections caused by *Acanthamoeba* are most common in patients with AIDS, with or without CNS involvement (69, 125, 179, 322). Cutaneous disease has also been documented for non-HIV-infected patients with amebic encephalitis, for patients undergoing immunosuppressive therapy for organ transplantation (421, 449), or for individuals with immunological diseases (159, 277, 337, 421, 460). The cutaneous form of the disease is characterized by the presence of hard erythematous nodules or skin ulcers (40, 77, 143, 157, 179, 258, 295, 322, 380, 438, 446). Early manifestations of the cutaneous form of acanthamoebiasis include the presence of firm papulonodules that drain purulent material and then develop into nonhealing indurated ulcerations (295, 380). Occurrence of disseminated skin lesions may be the presenting manifestation of *Acanthamoeba* infection (322, 438). Whether skin lesions represent a primary focus of infection or are the result of hematogenous dissemination from other sites such as the respiratory tract, sinuses, or the CNS is not known (143). The reported mortality rate from cutaneous infection for individuals without CNS involvement is approximately 73%, while that

TABLE 2. Differences among the free-living amebae causing disease in humans[a]

Characteristic	*Acanthamoeba*			*Balamuthia*	*Naegleria*
	GAE	AK	Cutaneous lesions, sinusitis	GAE	PAME
Portal of entry	Olfactory epithelium, respiratory tract, skin, sinuses	Corneal abrasion	Skin, sinuses, respiratory tract	Olfactory epithelium, skin, respiratory tract	Olfactory epithelium
Incubation Period	Weeks to months	Days	Weeks to months	Weeks to months	Days
Clinical signs	Confusion, headache, stiff neck, irritability	Blurred vision, photophobia, inflammation, corneal ring	Skin lesions, nodules, sinus lesions, sinusitis	Slurred speech, muscle weakness, headache, nausea, seizures	Headache, nausea, vomiting, confusion, fever, stiff neck
Pathology	Focal necrosis, granulomas	Ulceration of cornea	Granulomatous reaction in skin, inflammation	Multiple necrotic foci, inflammation, cerebral edema	Hemorrhagic necrosis
Diagnosis	Brain biopsy, CSF smear/wet prep, culture, IIF[b] of tissue, PCR	Corneal scrape, corneal biopsy, calcofluor white, culture of material, confocal microscopy	Skin lesion biopsy, culture, IIF of tissue	Brain biopsy, culture on mammalian cells, IIF of tissue	Brain biopsy, CSF wet prep, CSF culture, IIF of tissue, PCR

[a] Data from references 113, 286, and 387.
[b] IIF, indirect immunofluorescence.

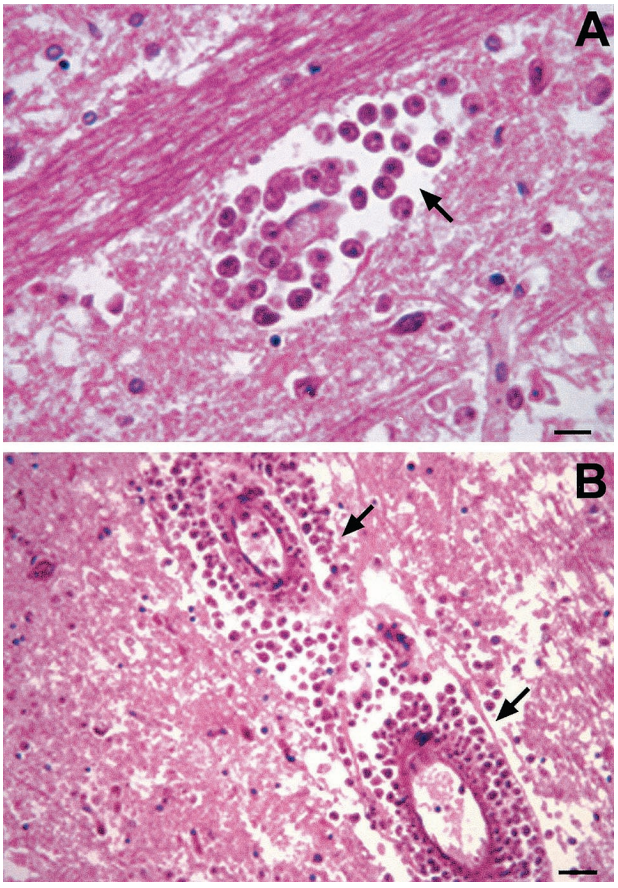

FIG. 7. H & E stain of brain tissue from a human with GAE. (A). Numerous trophozoites can be identified within the tissue (arrow). (B) Trophozoites identified within vascular walls (arrow). Bars, 150 μm (A) and 300 μm (B). Photographs courtesy of A. J. Martinez.

from cutaneous infection accompanied by CNS disease is 100% (446).

Histologic examination of cutaneous lesions generally shows foci of necrosis surrounded by inflammatory cells, vasculitis, trophozoite and cyst forms (143). However, the histologic appearance of skin lesions may mimic that of fungi, viruses, mycobacteria, or inflammation due to a foreign body (77, 159, 419). Organisms in tissue sections have been mistaken for yeast forms of *Blastomyces dermatiditis* (438), sporangia of *Rhinosporidium seeberi*, *Cryptococcus neoformans*, or *Prototheca wickerhamii* (420). Also, cases of cutaneous acanthamebiasis have been misdiagnosed as bacillary angiomatosis (214), cat scratch fever (420), *Penicillium marneffei* infection (69), Kaposi's sarcoma (77) or cells with cytomegalovirus inclusions (442). Thus, when a single biopsy specimen does not reveal trophozoites when *Acanthamoeba* is suspected, the examination should be repeated with a different specimen (307, 337).

Therapeutic options for treatment of cutaneous acanthamebiasis are not clearly established. Patients given combination treatments have shown improvement, but the majority have died (128, 192, 420). Therapy is less successful when CNS involvement occurs. However, successful treatments of cutaneous acanthamebiasis using itraconazole, pentamidine, 5-fluocytosine, and topical chlorhexidine gluconate and ketoconazole cream have been reported (179, 421).

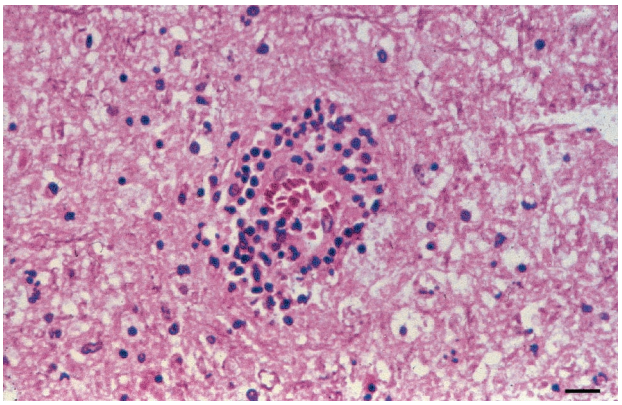

FIG. 8. H-&-E-stained section of paraffin-embedded brain tissue demonstrating granuloma formation in *Acanthamoeba* infection. Bar, 200 μm.

ACANTHAMOEBA SPP. AS NONOPPORTUNISTIC PATHOGENS

Amebic Keratitis

AK, first reported by Nagington et al. (325) in Great Britain and by Jones et al. (210) in the United States, is a painful progressive sight-threatening corneal disease (Fig. 9). Several species of *Acanthamoeba*, including *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. rhysodes*, *A. griffini*, *A. quina*, and *A. lugdunensis*, have been reported to cause AK (17, 315, 316, 394). Unlike debilitated patients with GAE or cutaneous acanthamebiasis, individuals with AK generally are immunocompetent. Nevertheless, these individuals do not develop protective immunity, and reinfection can occur (334). In the mid-1980s, an epidemic of AK occurred which was attributed to the increased use of contact lenses and poor lens hygiene (403, 404). *Acanthamoeba* organisms have been cultured from lens

TABLE 3. Signs and symptoms of *Acanthamoeba* infections[a]

Symptoms of infection of:	
CNS	Eyes (AK)
Headache	Eyelid ptosis
Mental status abnormalities	Conjunctival hyperemia
Seizures	Photophobia
Stiff neck	Watering (tearing)
Irritability	Blurred vision
Nausea and vomiting	Ocular pain
Hemiparesis	Corneal ring
Cranial nerve palsies	Perineural infiltrates
Hallucinations	Opacities
Gait ataxis	Loose corneal epithelium
Diplopia	Irritation
Photophobia	
Sleep disturbances	
Anorexia	
Babinski's sign	
Kernig's sign	

[a] Data from references 265 and 286.

TABLE 4. Treatment regimens and outcomes of *Acanthamoeba* infections

Patient no.	Underlying disease	Amebic disease	Therapy[a]	Outcome	Reference
1	AIDS	Sinusitis	Rifampin/KC	Died/septicemia	157
2	AIDS	Cutaneous	TMP/AmpB/clin/gentamicin	Died/GAE	474
3	AIDS	GAE	Pyrimethamine + flucon	Died/GAE	149
4	AIDS	Otitis	Chloramphenicol/AmpB/ceftizoxime	Died/GAE	114
5	Connective tissue	Cutaneous/vasculitis	Ampicillin/chloramphenicol/AmpB/ adenine arabinoside	Died/vasculitis	159
6	AIDS	GAE	Pyrimethamine/sulfadiazine	Died/GAE	159
7	AIDS	GAE	Pyrimethamine/sulfadiazine	Died GAE	159
8	AIDS	Sinusitis/cutaneous	KC/flucon + sulfadiazine	Died/GAE	143
9	AIDS	Cutaneous	AmpB + broad-spectrum antibiotics	Died/GAE	438
10	AIDS	Cutaneous	Flucon + sulfadiazine	Died/GAE?	438
11	AIDS	Cutaneous/sinusitis	Ketoconazole + IV FC	Resolution of lesions No CNS involvement	179
12	AIDS	Cutaneous lesions	AmpB + FC + flucon	Died GAE?	420
13	AIDS	Cutaneous lesions	KC + FC	Lesions resolved/died inanition	420
14	Renal transplant	Cutaneous lesions, IgA deficiency	Pentamidine + chlorhexidine + 2% KC cream; maintenance on oral IT	Lesions resolved, no CNS involvement	421
15	AIDS	Sinusitis + cutaneous	FC alone	Died	322
16	AIDS	Cutaneous + CNS	KC/pentamidine	No response	322
			Oral FC + IV pentamidine	Skin lesions improved-Died GAE	322
17	AIDS	Sinusitis + cutaneous	Pentam, KC, flucon, IT + metron + AmpB	Died	322
18	AIDS	Cutaneous/sinusitis	AmpB + rifamp + FC + IT + pentam	Multidrug toxicity, died	192
19	AIDS	Endophthalmic + cutaneous	5FC + pentam/propam/clotrim/neomycin eye drops	No response	178
20	AIDS	Cutaneous/sinusitis/otitis	FC	Minimal response	128
21	AIDS	Cutaneous	Oral IT + FC	Died	77
22	AIDS	Cutaneous	IV pentam + oral KC + flucon	Died	77
23	AIDS	Cutaneous	Prednisone + clin + TMP/SMX + flucon	Died GAE	173
24	AIDS	Cutaneous	TMP/SMX + flucon + rifabutin + pyrimethamine + sulfadiazine	Died GAE	173
25	AIDS	Cutaneous	Flucon + pentam + IT		
		Amebic osteomyelitis	FC and pentam, extended therapy for over 1 yr		407
26	AIDS	Cutaneous	Pentam + IT + chlorhexidine + topical KC	Pentamidine toxicity, Died sepsis	307
27	Lung transplant	Cutaneous	AmpB + IC + pentam + FC + chlorhex/KC cream + azithro + FC + pentam, maintenance FC + clarithromycin	Survived	337
28	AIDS	Cutaneous	5FC + oral IT	Died, inanition	69
29	AIDS	Sinusitis	IV pentam + oral IT + IV metron	Died/GAE	224
30	AIDS	CNS lesion	Sulfadiazine + pyrimethamine, flucon + sulfadiazine; one localized brain lesion excised	Survived	406
31	HIV positive	Sinusitis	Debridement of sinuses, IT + gentamicin nasal wash	Resolution of lesions	442
32	AIDS	Sinusitis/cutaneous	Debridement of sinuses + pentam + topical chlorhexidine + 2% KC; pentam toxicity, maintenance on FC	Survived	442
33	AIDS	Cutaneous	Pentam + flucon + azithromycin + topical econazole + chlorhexidine	Died	446
34	AIDS	Sinusitis/cutaneous	AmpB + pentam IV + FC + itraconazole + 2% KC cream; pentam toxicity, oral FC, oral IC + KC cream	Skin lesions healed, died of septicemic shock	258
35	CNS	Immunocompetent	TMP/SMZ + KC + rifampin	Survived	419
36	CNS	Immunocompetent	TMP/SMZ + KC + rifampin	Survived	419
37	HIV positive	Sinusitis/cutaneous, lobular panniculitis	Surgical debridement + IT, azithromycin, 5FC, rifampin	Survived	380
38	Lung transplant	Cutaneous	Pentam + IC + KC + chlorhexidine	Died	449
39	AIDS	Rhinosinusitis	Removal of all diseased nasal mucosa, pentam, levofloxacin, FC, AmpB	Survived	373

[a] FC, fluorocytosine; IT, itraconazole; azithro, azithromycin; AmpB, amphotericin B; KC, ketoconazole; TMP/SMZ, trimethoprim-sulfamethoxazole; pentam, pentamidine; flucon, fluconazole; metron, metronidazole; chlorhex, chlorhexidine; IV, intravenous; clin, clindamycin.

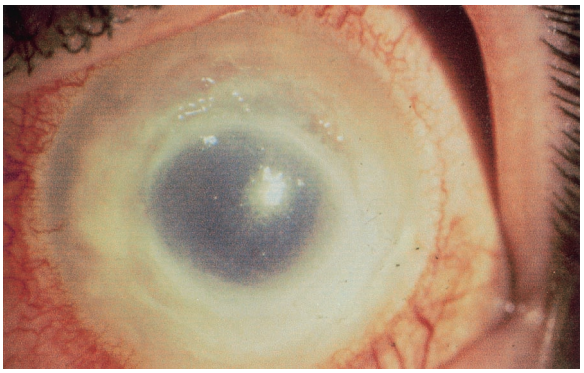

FIG. 9. Stromal infiltrate in AK. Photograph of a human with AK provided by P. C. Maudgal, Katholieke Universiteit Leuven, Leuven, Belgium.

cases and saline cleaning solutions. It is now recognized that the wearing of contact lenses is the leading risk factor for AK (116, 196, 425). Conditions which promote disease include the use of home-made saline solutions, poor contact lens hygiene, and corneal abrasions (16, 117, 195, 333, 409). Corneal trauma due to injury by a foreign body and exposure to contaminated water also may be associated with *Acanthamoeba* infection. Disease symptoms (Table 3) include redness, tearing, photophobia, and lid edema. The histopathologic picture of AK varies (237, 306). Initially, amebae are restricted to the corneal epithelium, but as the disease progresses, they invade the underlying stroma, cause extensive damage, and provoke mild to severe inflammation. The most characteristic clinical feature of AK is the presence of a ring-like stromal infiltrate, thought to be composed of infiltrating inflammatory cells such as neutrophils. Clinically, conjunctival hyperemia, corneal inflammation, episcleritis, and scleritis occur. Trophozoites can infiltrate corneal nerves, causing neuritis and necrosis (150, 288). In rare circumstances, *Acanthamoeba* can spread from the cornea to the retina, causing chorioretinitis (208, 319). In severe cases which do not respond to medical and surgical therapy, enucleation of the eye may be required (118). An in-depth discussion of the clinical progression of AK has been presented by Illingworth and Cook (195).

AK can be difficult to diagnose and treat. It has been diagnosed mistakenly as atypical herpes simplex keratitis or fungal keratitis (294, 394, 440). Diagnosis is complicated by the frequent occurrence of secondary bacterial infections. Antibacterial, antiviral, antifungal, or corticosteroid treatment may complicate the diagnosis because there is initial improvement following application of these therapeutic approaches which is followed by a worsening of the disease. Proper diagnosis is important for early treatment, since amebic cysts which form in tissues are resistant to many drugs.

Since contaminated contact lens cases and cleaning solutions are a source of infection, decontamination of lens cases and storage solutions is essential for preventing *Acanthamoeba* infection. A 3% solution of hydrogen peroxide has been used for disinfection of contact lenses and lens cases because it has been shown to be active against cysts and trophozoites (191). Hiti et al. (182) evaluated commercially available contact lens storage solutions for amebicidal activity. It was indicated that a two-step hydrogen peroxide system (0.6% H_2O_2) was an effective disinfectant after an 8-h soaking of contact lenses and lens cases. However, two of the three strains used to test cysticidal activity were not those typically associated with AK. Zanetti et al. (486) demonstrated that a 1:2 dilution of a 3% hydrogen peroxide solution killed cysts of *A. castellani* after a 9-h exposure. It has been recommended that a two-step hydrogen peroxide system be used at concentrations of 3% rather than 0.6% since bacteria present in lens cases produce catalase, which neutralizes the peroxide (30). Microwave irradiation also has been reported to effectively kill *Acanthamoeba* spp. after 3 min of treatment (181). Pinna (354) recommended the use of "1-day" disposable contact lenses to reduce the risk of keratitis.

DIAGNOSIS OF *ACANTHAMOEBA* INFECTIONS

Granulomatous Amebic Encephalitis

For patients who present with CNS symptoms, diagnosis of *Acanthamoeba* can include direct microscopy of wet mounts of CSF or stained smears of CSF sediment (64, 88, 245, 419). However, while assessment of CSF may be of value in the diagnosis of GAE, lumbar puncture may be contraindicated because of increased intracranial pressure (265, 286). CSF is centrifuged at low speed (250 × *g*) for 10 min to avoid rupture of trophozoites (265). Trophozoites may be observed in wet preparations of CSF but may be unrecognized because they resemble macrophages. CSF sediment can be smeared on a glass slide, fixed in methanol, and stained with Giemsa-Wright stain. Pleocytosis with abundant lymphocytes and polymorphonuclear leukocytes and low glucose levels with high protein levels are suggestive of GAE. CSF or bronchoalveolar lavage fluid cytospin preparations also have been used to identify amebae in patients with GAE or respiratory illnesses (31, 330). However, certain characteristic features of *Acanthamoeba* trophozoites such as a prominent nucleolus, contractile vacuole, and cytoplasmic vacuoles may be visualized more readily using trichrome or hematoxylin and eosin (H & E) stains on fixed preparations after cytocentrifugation rather than using air-dried preparations (330).

Computed tomography and magnetic resonance imaging have been performed on some patients (149, 286). These procedures have shown that while enhancing lesions are present in many individuals, nonenhancing lesions are present in others. Computed tomography or magnetic resonance imaging of the brain may reveal multifocal areas of signal intensity or discrete lesions suggestive of abscesses or brain tumors (218, 291, 397, 408). However, although imaging analysis reveals CNS abnormalities, it does not provide a definitive diagnosis of GAE. In fact, amebic meningoencephalitis has been misdiagnosed as neurocysticercosis based on neuroimaging findings (291).

Methods for in vitro cultivation of *Acanthamoeba* also have been used for laboratory diagnosis. CSF, brain tissue, or material from cutaneous or sinus lesions can be inoculated into ameba growth medium, onto NNA plates containing a layer of *Escherichia coli* or *Enterobacter aerogenes* cells, or onto monolayers of cultured mammalian cells (285). Clear plaques are observed after a week of growth where amebae have ingested bacteria or cells. However, isolation of *Acanthamoeba* from

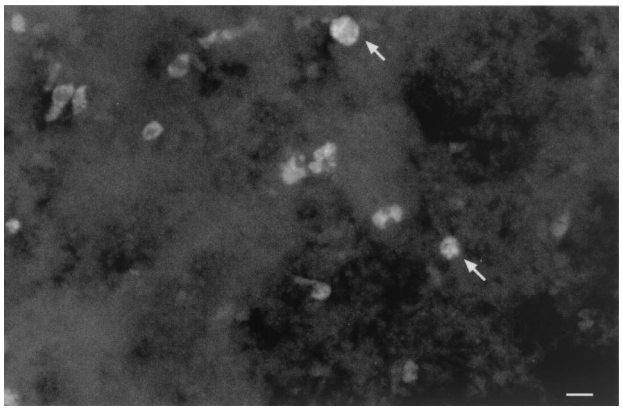

FIG. 10. Calcofluor white fluorescent staining of a mouse brain section to identify trophozoites and cysts in infected tissues. Calcofluor white has been used for the identification of cysts (arrow) in cases of AK and can be used to identify cysts in brain tissue or cutaneous lesions. Bar, 50 μm.

CSF or infected tissues is difficult because many of the amebae may be encysted (222).

In addition, histological diagnosis can be made on the basis of frozen or paraffin-embedded sections of brain or cutaneous lesion biopsy material stained with H & E (Fig. 7) (286, 380, 406, 442). Although Gram, Giemsa, and H & E staining are not differential, macrophages and other immune cells can be distinguished from ameba trophozoites based on nuclear morphology. The nuclear structure of *Acanthamoeba* is characterized by a pronounced karyosome and surrounding "halo," which is completely unlike that for any inflammatory cell. However, several staining methods have proved useful for identification of cysts in tissue sections. Periodic acid-Schiff stains the cyst wall red, while Gomori-methenamine silver stains the cyst black. In addition, calcofluor white (Fig. 10) has been used to identify cysts in brain tissue (414). Microscopic findings in infected tissue stained with H & E generally reveal granulomas (Fig. 8) with multinucleated giant cells, but these may be absent in immunosuppressed individuals. H-&-E-stained tissues (Fig. 7) reveal focal necrosis and amebic trophozoites and cysts throughout lesions which are located primarily in perivascular spaces and invading blood vessels (279).

Immunofluorescent or immunoperoxidase cytochemical staining of cryostat sections or infected tissues embedded in paraffin, as well as transmission electron microscopy of infected tissues, has been employed for identification of *Acanthamoeba* with greater success (Fig. 11) (265, 428, 461, 476). Use of specific antibodies to different species of *Acanthamoeba* in conjunction with immunofluorescent staining has allowed the identification of amebae in tissue sections. However, while organisms can be identified as members of the genus *Acanthamoeba*, discrimination of distinctive species is difficult since many species are antigenically related (285).

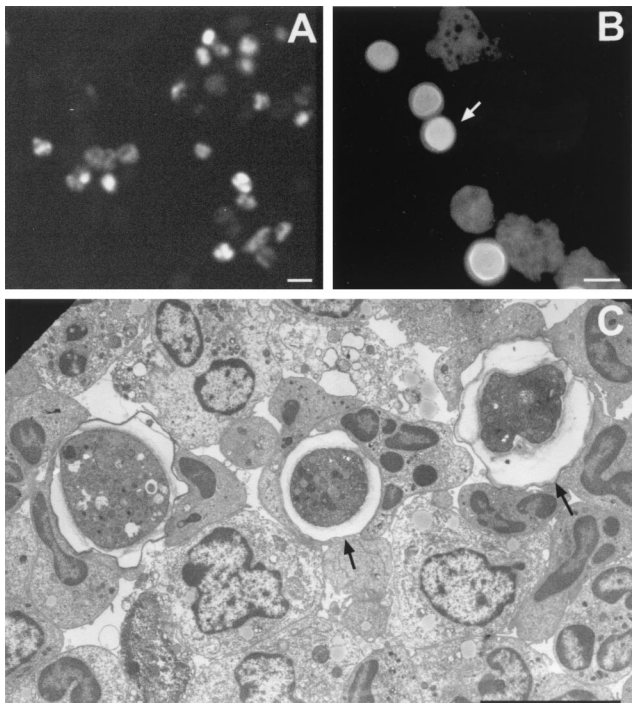

FIG. 11. Micrographs illustrating diagnostic methods used to identify *Acanthamoeba* in infected tissues. (A) Immunofluorescence of trophozoites using anti-ameba antibodies; (B) calcofluor white staining of trophozoites and cysts (arrow) of *Acanthamoeba*. (C) Electron micrograph of material from infected mice. Electron microscopy has been used to identify cysts (arrows) and trophozoites more readily in infected tissues. Bars, 50 μm (A), 25 μm (B) and 10 μm (C).

Amebic Keratitis

Early detection and diagnosis is critical to the outcome of the clinical course of AK infections. A diagnosis of AK should be considered when chronic corneal ulcers are unresponsive to antibiotic therapy. However, ocular infections with *Acanthamoeba* are difficult to diagnose because they can resemble those due to herpes simplex virus, *Pseudomonas aeruginosa*, or fungal infection. As a result, there is often a significant delay in formulating an appropriate diagnosis before treatment is started. Corneal or conjunctival swabs are generally not suitable for isolation of *Acanthamoeba* (482). Corneal scrapes or corneal biopsy specimens are used for culture or for identification of cysts or trophozoites in stained tissue sections (108). For culture, material from a corneal scrape can be placed onto nonnutrient agar containing *E. coli* or inoculated into liquid medium (174, 220). However, corneal scrapes may contain bacteria or yeast, which can confuse the diagnosis (16, 394). *Acanthamoeba* has been cultured from contact lenses, lens cases, and lens-cleaning solutions when cultured corneal tissues were negative (15, 196, 314). A positive culture of the lens case or cleaning solution does not confirm the diagnosis but suggests infection with *Acanthamoeba* (329). In a retrospective study in which records were examined for amebae in clinical specimens, *Acanthamoeba* were recovered from approximately 73% of clinical specimens inoculated onto commercially available buffered charcoal-yeast extract agar, 71% of specimens inoculated onto NNA with *E. coli*, and 70% of specimens inoculated onto Trypticase soy agar containing horse or sheep blood (349). Other investigators have reported less satisfactory results using corneal scrapings to culture *Acanthamoeba* (196, 290, 440). NNA plates seeded with *E. coli* and inoculated with

corneal specimens should be incubated at 28 to 35°C and held for an extended interval (10 days or more to ensure time for excystment) because some species of *Acanthamoeba* do not grow well at 35°C or above (222). Corneal biopsy has been suggested when repeated cultures of corneal scrapings are negative (16, 17). For cytological diagnosis, various staining methods can be employed. The indirect immunofluorescent-antibody assay (Fig. 11A) has been used to detect amebae in corneal scrapings or in biopsy tissue (131). Calcofluor white, a chemofluorescent dye with an affinity for the polysaccharide polymers of amebic cysts, has been used to identify amebic cysts in corneal tissue (475). Calcofluor white stains amebic cyst walls bright apple green, and this effect can be enhanced by prolonging the staining period (Fig. 11B). Evans blue is used to counterstain the background (475). Trophozoites and cysts in paraffin-embedded tissues can also be rapidly and differentially stained with calcofluor white (273, 414). In addition, acridine orange staining of corneal scrapings or CSF has been recommended as a simple and reliable method for rapid histological diagnosis of AK or GAE (100, 167).

In addition to various staining methods, the usefulness of PCR for detection of *Acanthamoeba* has been demonstrated, although a number of probes which have been developed are not species specific (189, 216, 223, 231, 256, 289, 462). A procedure based on application of a nonradioactive DNA probe prepared from a variable region of cloned 26S rDNA in concert with PCR has been used for the specific detection of *Acanthamoeba.* Using this technique, as few as 10 *Acanthamoeba* cells could be detected (243). In addition, a method which employs a PCR primer pair which produces an amplimer that is specific for the genus *Acanthamoeba* has been used for detection of *Acanthamoeba* in environmental samples and corneal scrapings from AK patients (129, 251, 395, 434). More recently, a promising novel means of identification of *Acanthamoeba* in clinical specimens was reported which consists of fluorescence in situ hybridization using a genus-specific probe or a sequence type 4 (T4)-specific probe to enhance the detection of *Acanthamoeba* (433). In this procedure, the fluorescein-labeled 22-mer genus-specific probe hybridizes specifically to all *Acanthamoeba* 18S rDNA sequence types but does not react with *Balamuthia mandrillaris* or *Hartmanella vermiformis.* To date, a T4 probe has been used because most species which have been identified as associated with AK belong to sequence type T4. Results can be obtained in 1 to 2 days without the need for culturing the organisms, which could take 1 to 2 weeks or longer. However, when negative results are obtained with corneal scrapings by fluorescence in situ hybridization, it has been recommended that these results be confirmed by culturing the corneal sample (433). Additionally, Lehmann et al. (256) have reported the use of a PCR assay to detect *Acanthamoeba* DNA in corneal and tear specimens containing as few as one to five amebae. These investigators suggested that a PCR assay of tears not only could serve as a diagnostic tool but also could be used to monitor the response to treatment.

A variety of molecular methods of identification of *Acanthamoeba* in samples have been employed. These consist of analysis of DNA sequence variation through RFLPs of complete or partial nuclear 18S rRNA gene sequences (83, 225, 231, 234, 437), variation in complete mitochondrial 16S rRNA (83, 231) or the complete mitochondrial genome (62, 154, 219, 234, 483, 483), analysis of DNA sequences of complete or partial DNA fragments coding for 18S rRNA (129, 256, 434, 466), and randomly amplified polymorphic DNA analysis of whole-cell DNA (10). Immunodiagnostic probes for identification of *Acanthamoeba* also have been employed. A bacteriophage antibody display library has been used to isolate antibody fragments that bind specifically to *Acanthamoeba* spp. in specimens by immunofluorescence or flow cytometry (215). Finally, several investigators have reported the use of tandem scanning confocal microscopy, a noninvasive technique, for in vivo diagnosis of AK. Corneal examination by scanning confocal microscopy has been associated with an increase in the detection of *Acanthamoeba* (6, 80, 290, 352). The confocal microscope allows the visualization of high-contrast images of coronal corneal sections containing trophozoites or cysts on a video monitor. Thus, a number of rapid techniques are now available for the detection of *Acanthamoeba* in tissues. These methods should be considered when formulating a laboratory diagnosis so that treatment can be started as soon as possible.

Cutaneous Acanthamoebiasis

Material from cutaneous lesions can be inoculated into ameba growth medium, onto NNA plates containing *Escherichia coli* or *Enterobacter aerogenes*, or onto mammalian cell culture monolayers and assessed for ameba growth as described for the laboratory diagnosis of GAE. Histological diagnosis of cutaneous lesion biopsy material can be performed using H & E, periodic acid-Schiff, or calcofluor white staining procedures. In addition, DNA-based molecular methods such as RFLP and randomly amplified polymorphic DNA analysis, as well as immunocytochemical approaches, can be applied.

TREATMENT OF *ACANTHAMOEBA* INFECTIONS

Disseminated *Acanthamoeba* Infections

A number of therapeutic agents and plant extracts have been tested in vitro for amebicidal activity against pathogenic *Acanthamoeba* spp. (Table 4). However, conflicting results have been reported. Ketoconazole, pentamidine, hydroxystilbamidine, paromomycin, 5-fluorocytosine, polymyxin, sulfadiazine, trimethoprim-sulfamethoxazole, azithromycin, and extracts of medicinal plants have been indicated as being active against *Acanthamoeba* in vitro, but no direct evidence has been obtained that these agents are efficacious in individuals with GAE (49, 67, 82, 126, 339, 377, 398, 400, 401, 429, 444). Therapeutic agents have also been tested in experimental animals. In mice, sulfadiazine, rifampin and flucytosine are effective against *Acanthamoeba* if administered before or within 24 h of exposure (107, 383).

In human infections, combination therapies have proven more successful than single-drug therapies because many drugs exhibit amebostatic but not amebicidal activity. No single drug has yet been shown to be effective against both the trophozoite and cyst stages of *Acanthamoeba.* Furthermore, *Acanthamoeba* infections are not readily recognized by clinicians or pathologists because many patients present with underlying disease (269). Depending on the immune status of the host, infection with *Acanthamoeba* spp. can result in dissemination to the skin,

lungs, CNS, and other organs. Therefore, increased awareness of the potential for infection with *Acanthamoeba* is important for early detection and treatment. Early treatment is important because most patients with disease disseminated to the CNS die. In early reports of amebic encephalitis, corticosteroid therapy was instituted because of cerebral edema and inflammation (164, 422). However, it has been suggested that steroids exacerbate *Acanthamoeba* infection and should not be used (96, 274, 276, 283, 298).

Reports of successful treatment of *Acanthamoeba* infection have been few (Table 4). Ketoconazole and rifampin added to trimethroprim-sulfamethoxazole therapy was used successfully for treatment of two immunocompetent pediatric patients with CNS infection (419). Slater et al. (421) reported successful treatment of disseminated *Acanthamoeba* infection in a renal transplant patient who was HIV negative. Therapy consisted of a 4-week course of IV pentamidine isethionate, topical chlorhexidine gluconate, and 2% ketoconazole cream. The success of this therapeutic regimen was attributed to early treatment before onset of CNS infection. Resolution of cutaneous lesions and sinusitis with 5-fluorocytosine treatment in a patient with HIV infection without CNS involvement has also been reported (179). Seijo et al. (406) reported a successful outcome in an HIV-positive individual with CNS involvement. Surgical removal of one localized CNS lesion followed by therapy with fluconzole and sulfadiazine resulted in effective treatment and survival of the patient. Teknos et al. (442) suggested the use of surgical debridement of nasal and paranasal sinuses as soon as possible after identification of organisms in nasal tissue, followed by long-term therapy. These investigators recommended 5-fluorocytosine for CNS infection and for renal transplant patients rather than pentamidine because of the nephrotoxicity of the latter compound and the fact that it does not cross the blood brain barrier. Nephrotoxicity caused by pentamidine isethionate treatment has been reported in other patients with *Acanthamoeba* infection (307, 449). Rivera and Padhya (373) successfully treated an AIDS patient with rhinosinusitis by surgical removal of all diseased areas of the nasal mucosa followed by prolonged therapy with pentamidine, amphotericin B, flucytosine, rifampin, itraconazole, and chlorhexidine.

Treatment with multidrug regimens for humans with disseminated *Acanthamoeba* infections have met with mixed results. An HIV-positive patient with recurrent sinusitis and cutaneous lesions was stabilized after 7 weeks of treatment with itraconazole, azithromycin, 5-fluorocytosine and rifampin (380). Multidrug therapy was used successfully in a lung transplant recipient without CNS involvement (337). On the other hand, multidrug treatment regimens have proven less successful for some patients because of the induction of multidrug toxicity. Levine et al. (258) attempted multidrug therapy in an HIV-positive patient with cutaneous acanthamoebiasis. Use of a combination of amphotericin B, rifampin, and 5-fluorocytosine resulted in initial improvement but the patient died of gram-negative bacterial septicemia. Hunt et al. (192) treated lesions with a combination of amphotericin B, rifampin, and 5-fluorocytosine, followed by itraconazole, rifampin, and 5-fluorocytosine after recurrence of disease. However, treatment was not successful and the patient died. While many patients respond to initial treatment, a number of these die of related illnesses (69, 128, 258, 307, 322). Thus, to date, the collective data which have been obtained indicate that effective treatment is predicated on early diagnosis and initiation of treatment before dissemination of amebae to the CNS.

Cutaneous Acanthamoebiasis

In patients who are immunocompromised because of HIV infection or patients undergoing immunosuppressive treatment for organ transplantation, cutaneous *Acanthamoeba* infections with dissemination to other organs has been reported (337). Patients with cutaneous acanthamoebiasis have been treated with various drugs. Helton et al. (179) reported a successful treatment outcome using 40 mg of 5-fluorocytosine per kg for 2 weeks in an AIDS patient with cutaneous and sinus lesions. Therapy with intravenous pentamidine and itraconazole along with topical ketoconazole and chlorhexidine was reported by Van Hamme et al. (449) to be ineffective in a lung transplant patient, who developed cutaneous lesions and died. Treatment with pentamidine, ketoconazole, fluconazole, itraconazole, metronidazole, and amphotericin B consecutively did not resolve lesions in an HIV-positive patient with sinusitis and cutaneous lesions. Cardiac toxicity due to pentamidine treatment was noted in another patient with cutaneous disease (258).

Amebic Keratitis

Because diagnosis is difficult and treatment is often delayed, infection with *Acanthamoeba* may result in total loss of sight in the infected eye. If infection is recognized early, wide epithelial debridement may be curative if the epithelium alone is involved (53, 184, 261). Debridement may remove infectious organisms and enhance the delivery of topical medications (17, 198). When *Acanthamoeba* infection proceeds without early treatment, the organisms invade deeper into the corneal region. Under these circumstances, when therapy is instituted it is often continued for several months to 1 year or longer (17). Furthermore, patients must be monitored for recurrence of disease because cysts are resistant to many drugs. Treatment failures are frequently reported; this may be attributed to poor penetration of the agent, insufficient duration of treatment, or acquired resistence to the drugs. A number of therapeutic agents are not effective at the late stage of infection, especially when the amebae have invaded tissues beneath the cornea (118, 139, 161, 186, 323, 447). Binder (36) suggested cryotherapy for patients showing a poor response to medicinal and surgical treatment.

In vitro susceptibility testing of isolates may prove beneficial for application of early treatment regimens. An in vitro susceptibility test for *Acanthamoeba* isolated from AK patients has been developed (326). Isolates are grown for 1 week on NNA seeded with *E. coli* to elicit encystation. The cysts are then scraped from the agar, washed, and incubated in dilutions of anti-ameba drugs for 48 h. Following removal of drugs, the cysts are placed on fresh NNA plates to assess the growth of amebae. The minimal cysticidal concentration is determined and is defined as the lowest concentration of test solution that results in no excystment and growth of trophozoites after 7 days of culture.

Wright et al. (482) reported successful treatment of AK

using 0.1% propamidine isethionate (Brolene) topically with 0.15% dibromopropamidine. This treatment regimen was effective only when initiated early in infection (17, 120, 294, 314, 402, 482). In vitro studies have demonstrated that trophozoites of *Acanthamoeba* isolated from AK patients are susceptible to chlorhexidine and propamidine (402). Topical administration of these two drugs was found to be effective for treating AK, provided that the drugs were given for extended periods. However, propamidine is not recommended for all cases since some patients with AK have developed corneal abnormalities following prolonged treatment (206, 323). A successful outcome has been reported for patients with superficial AK treated with 0.1% hexamidine (51). A series of 12 patients with culture-proven AK were monitored during and after therapy with topical chlorhexidine and propamidine. Chlorhexidine in combination with propamidine provided rapid and successful treatment for corneal *Acanthamoeba* infections (402). Kosrirukvongs et al. (236) reported that four of five culture-proven AK patients were treated successfully with 0.006% chlorhexidine solution alone.

Imidazoles such as miconazole, itraconazole, and ketoconazole have been used with limited success (32, 108, 186, 198, 261, 315, 482). Topical treatment with miconazole, however, has lead to epithelial toxicity. Ketoconazole is more effective for treatment of AK but must be given systemically. Polyhexamethylene biguanide (PHMB), manufactured as an environmental disinfectant known as Baquacil by Zeneca Pharmaceuticals, was shown to exhibit both amebicidal and cysticidal activity against a number of *Acanthamoeba* strains (405). Although not licensed for therapeutic use, it has been employed as a successful experimental treatment for AK by Larkin et al. (250). A combination of chlorhexidine digluconate (a bisbiguanide) with PHMB (a polymeric biguanide) or with aromatic diamidines such as hexamidine, pentamidine, and propamidine isethionate or PHMB and hexamidine with debridement has been reported for the treatment of AK (124, 161, 171, 175, 195, 196, 250, 260, 323, 365, 405, 447, 454, 482). PHMB alone does not appear to be associated with toxicity, but other compounds used in combination with PHMB may exert untoward effects (195). Recently, a patient with unconfirmed but suspected AK was reported to have developed progressive ulcerative keratitis related to the use of topical chlorhexidine gluconate for 8 weeks (324). Penetrating keratoplasty was performed because of the possibility of corneal perforation. However, the usefulness of penetrating keratoplasty in the treatment of AK has been debated (17, 89, 180, 261, 394), and it has been used as a last resort for some patients. Penetrating keratoplasty may not be required if AK patients are treated within 6 weeks of presentation (196). Bacon et al. (17) indicated that this procedure is more successful if performed after resolution of inflammation. Cremona et al. (94) reported that deep lamellar keratectomy with a conjunctival flap was effective for controlling infection and relieving pain in two patients with advanced keratitis.

Corticosteroids have been used in conjunction with therapeutic agents for the treatment of AK (16). Although corticosteroids reduce inflammation, recent studies suggest that the use of corticosteroids should be avoided if possible (108). In vitro and in vivo studies using experimental animals suggest that exposure of cysts to corticosteroids such as dexamethasone phosphate increases the pathogenicity of the amebae (298). Keratitis in dexamethasone-treated hamsters was found to be more severe than in untreated animals. Additionally, locally administered corticosteroids were shown to be detrimental in a rabbit model of AK (205).

New therapeutic agents are being sought for the treatment of *Acanthamoeba* infections. More recently, the emergence of resistance to commonly used antimicrobial agents and biocides in the treatment of *Acanthamoeba* keratitis and in contact lens disinfection systems has been assessed (323, 448). Resistance to biocides apparently results from the physical barrier of the cyst wall rather than being a consequence of the presence of metabolically dormant cysts (448). In one study, the amebicidal activity of eight different alkylphosphocholines against *Acanthamoeba* spp. was investigated (182). Treatment with hexadecylphosphocholine resulted in complete lysis of *Acanthamoeba* in vitro within 1 h of addition of the compound. Although alkylphosphocholines are in the experimental stage of development as amebicidal agents, hexadecylphosphocholine may be useful for treatment of GAE as well as AK since this drug has been shown to cross the blood-brain barrier in experimental animals. Table 5 summarizes treatment regimens and outcomes for AK.

IMMUNOLOGY AND PATHOLOGY OF *ACANTHAMOEBA* INFECTIONS

Role of the Immune System in *Acanthamoeba* Infections

The immune defense mechanisms that operate against *Acanthamoeba* have not been well characterized. Exposure to *Acanthamoeba* appears to be common since the presence of antibodies to the ameba have been demonstrated in serum samples (Fig. 12) from most asymptomatic healthy individuals (73, 78, 104, 271, 330, 357). Nevertheless, although the prevalence of *Acanthamoeba* cysts and trophozoites is high in the environment, the incidence of fatal infection appears low. Indeed, *Acanthamoeba* have been isolated from the nasopharynx of apparently healthy individuals (19, 73, 75, 304, 330, 370, 371). Whether *Acanthamoeba* causes transient infections in these individuals and stimulates host defense responses which control infection and result in the elimination of the organism is not known. Chappell et al. (78) suggested that while serious ocular disease and CNS infections are rare, mucosal infections may contribute significantly to large numbers of undiagnosed sinus or pulmonary infections. The presence of a higher frequency of *A. polyphaga*-specific immunoglobulin M (IgM) and a lower frequency of IgG antibodies in serum from rheumatoid arthritis (RA) patients than from matched controls has been reported (204). The higher titers of *A. polyphaga*-specific IgM in RA patients than in matched controls were thought to be due to persistent or repeated antigenic stimulation by *A. polyphaga.* However, it is not known whether an immune reaction to *A. polyphaga* antigens results in symptoms in RA patients.

Protection from lethal infection may involve both innate and acquired immunity (103, 104). In experimental animal infections, the age of the animal, the mouse strain, the immune status of the host, the infecting dose, temperature tolerance, and the virulence of the ameba strain appear to be important

TABLE 5. Treatment regimens and outcomes for AK

Patient no.	Therapy[a]	Outcome	Reference
1	Propamidine isethionate, Neosporin	Toxicity	482
2	PHMB[b]	Successful	250
3	1% clotrimazole eye drops		
	Dibromopropamidine isethionate	Recurrence	118
	Oral fluconazole, clotrimazole, PHMB	Progression to enucleation	
4	Propamidine isethionate, neosporin	Treatment failure	161
	Miconazole, PNA, itraconazole	Toxicity	
	PHMB	Resolution of disease	
5	Miconazole, metronidazole, PNA, neomycin, ketoconazole	Residual infiltrate	186
6	Miconazole, metronidazole, propamidine, ketoconazole	Recurrence	186
7	Hexamidine	Well tolerated cure	51
8	PHMB and propamidine isethionate; steroids only after clinical improvement	Cure, long-term treatment necessary	196
9	Chlorhexidine and propamidine	Effective if used for the long term	402
10	Itraconazole, miconazole	Unresponsive	323
	Penetrating keratoplasty	Successful	
11	Propamidine, neosporin, itraconazole, miconazole	Corneal perforation, keratoplasty	323
12	Propamidine, PHMB	Resolution of disease	323
13	PHMB, propamidine, PNA, chlorhexidine, PHMB	Propamidine toxicity	323
14	Chlorhexidine, PHMB, propamidine isethionate	Treatment of choice	260

[a] Therapeutic agents reported for individual cases and treatment outcomes. PNA, prednisolone acetate.
[b] PHMB is not licensed for therapeutic use. It is used as experimental drug.

factors in the outcome of a murine infection (270). Complement, an innate resistance factor which provides the first line of defense against invading organisms, is activated by *Acanthamoeba* infection (138, 445). However, the consequences of such activation in response to infection with *Acanthamoeba* are not known. Whether complement activation results in increased pathogenesis by generating C3a and C5a components, which act as mediators of inflammation and tissue damage, or aids in eliminating amebae remains to be defined. Activation of complement may result in generation of opsonic factors such as C3b, which plays a role in recognition of amebae by phagocytic cells (138). Alternatively, complement may protect the host by lysing amebae, although in vitro, highly pathogenic species of *Acanthamoeba* such as *A. culbertsoni* are more resistant to complement lysis than are nonpathogenic *Acanthamoeba* spp. (445). It has also been shown that *Acanthamoeba* interacts with human C1q and that pathogenic strains appear to bind C1q more efficiently than nonpathogenic strains do. Binding of C1q by amebae blocked binding sites for C1, the first component of the classical complement cascade, which serves to inhibit the classical pathway (469). Thus, resistance to complement lysis may constitute a mode of immune evasion which contributes to the establishment of infection and dissemination of *Acanthamoeba* within the host (445, 469).

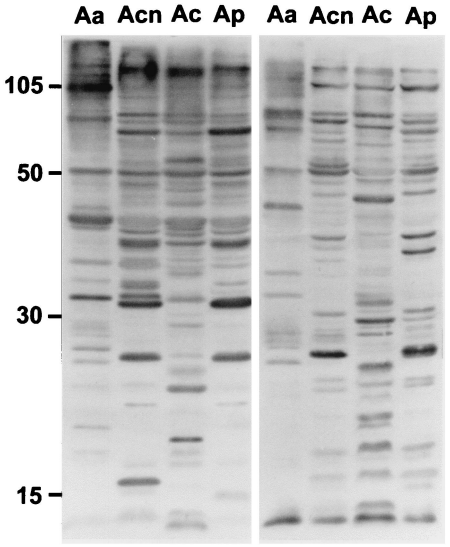

FIG. 12. Western immunoblot of *Acanthamoeba* whole-cell lysates reacted with normal human serum, demonstrating immunoreactivity against *Acanthamoeba* antigens. Serum samples from two asymptomatic individuals served as a source of natural antibodies to four species of *Acanthamoeba.* Whole-cell lysates of *A. astronyxis* (lanes Aa), *A. castellanii* (lanes Acn), *A. culbertsoni* (lanes Ac), and *A. polyphaga* (lanes Ap) were prepared and used as the antigen source. *Acanthamoeba* protein (50 μg) was added to each well of a sodium dodecyl sulfate–12% polyacrylamide gel. Separated proteins were electrophoretically transferred to a nitrocellulose membrane and were incubated with normal human serum as the source of primary antibody and horseradish peroxidase-labeled goat anti-human IgG as the secondary antibody. The blots were then subjected to enhanced chemiluminescence.

The role of antibodies in *Acanthamoeba* infection also remains unresolved. Antibodies may prevent attachment to host cells, inhibit the motility of amebae, or neutralize ameba cytotoxic factors (104, 136, 272, 431). However, it has been reported that *Acanthamoeba* can degrade human IgG and IgA antibodies by serine proteases (233). In mice, immunization with *A. culbertsoni* antigens using intranasal, intraperitoneal, intravenous, or oral routes of administration purportedly provided protection against a lethal challenge (382). However, multiple immunizations were required to impart protection. Of the immunogens tested (e.g., culture fluid, amebic sonicate, freeze-thawed extract, or live amebae), amebic sonicate elicited the best protection against intranasal challenge with *A. culbertsoni.* Protection was specific in that immunization with

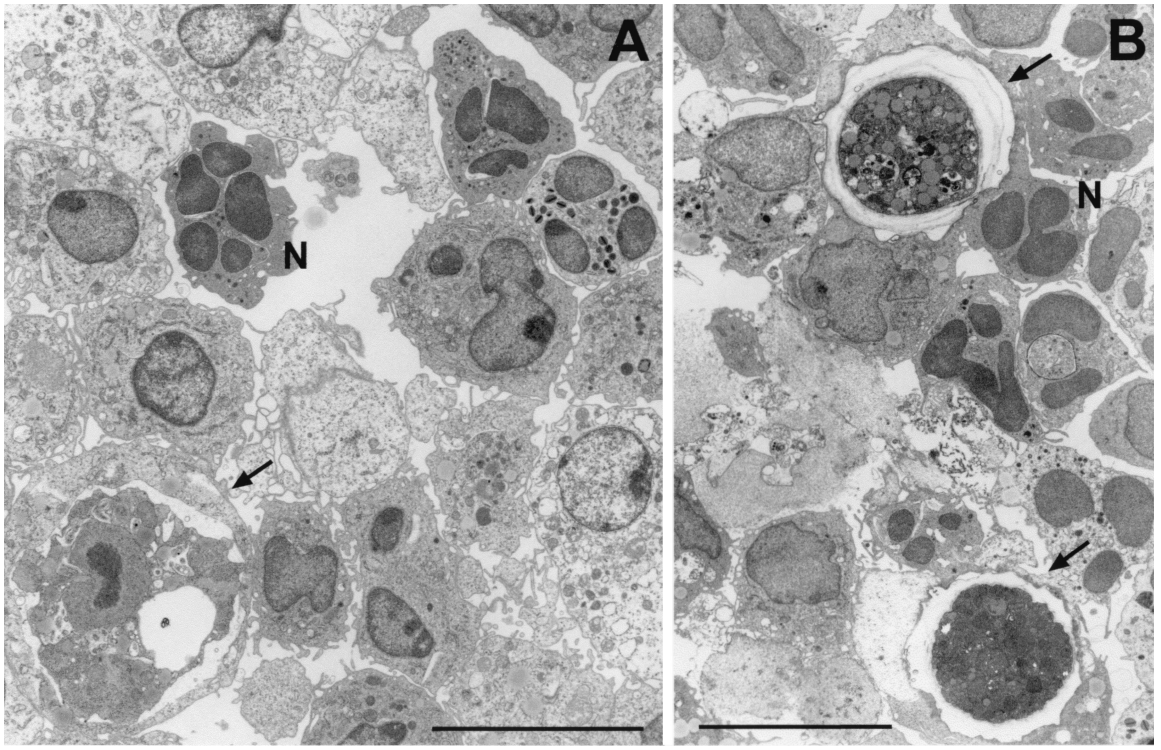

FIG. 13. Transmission electron micrographs demonstrating cellular events in an experimental mouse model of infection with *Acanthamoeba.* (A) Focal area of infection containing amebae after a 24-h exposure to *A. castellanii.* (B) Focal area of infection containing amebae after a 48-h exposure to *A. castellanii.* Neutrophils (N) are prominent in focal areas containing amebae (arrow) during the early phase of infection. Bars, 10 μm.

other species of *Acanthamoeba* did not protect mice against challenge with *A. culbertsoni* (34, 96, 135).

There have been few studies to assess the interaction of *Acanthamoeba* with specified cells of the immune system. It has been reported that the earliest response of the host to amebae consists of an influx of neutrophils to the site of infection (136). However, human neutrophils fail to kill *Acanthamoeba* unless the neutrophils are treated with TNF. In vitro, killing of *Acanthamoeba* by lymphokine-treated neutrophils requires the presence of both antibodies and complement (136). Studies in our laboratory with experimental animals such as mice exposed to *Acanthmoeba* have shown that neutrophils and macrophages are the major inflammatory cells to migrate into the area of infection. Macrophages may play a more important role than neutrophils in killing *Acanthamoeba* (Fig. 13 and 14). These cells are capable of injuring amebae and comprise the major cellular component of granulomas frequently encountered in tissues containing *Acanthamoeba* cysts (Fig. 14) (272). Masihi et al. (287) studied the effect of the mycobacterium-derived immunopotentiating agents muramyl dipeptide and trehalose dimycolate against intranasal *Acanthamoeba* infections in mice. Treatment of mice with these macrophage-activating agents prior to infection protected 40 and 30% of the animals, respectively, against a lethal infection with *A. culbertsoni.* In vitro studies with murine macrophages activated in vivo with *Bacillus* Calmette-Guérin demonstrated that activated macrophages were more efficient in injuring *Acanthamoeba* than were unstimulated macrophages (272). The activated macrophages also were more efficient in injuring *Acanthamoeba* than were unstimulated macrophage-like cells maintained as continuous cell lines. TNF-α and IL-1α or IL-1β, cytokine products of activated macrophages, were found not to be amebicidal for *Acanthamoeba* when used either alone or in combination (272). Hydroxy radicals, hydrogen peroxide, and nitric oxide may be important amebicidal factors since it has been reported that *Acanthamoeba* strains are sensitive to hydrogen peroxide (134). In addition, Stewart et al. (430) reported that rat macrophages, similar to murine macrophages, undergo chemotaxis to amebae and kill trophozoites in vitro. Thus, although the full range of specific macrophage factors responsible for injuring *Acanthamoeba* has yet to be defined, it is apparent that macrophages activated with immunomodulators are capable of phagocytizing and destroying amebae (272).

Recent reports indicate that microglial cells, resident macrophages of the brain, also exert amebicidal activity (271). Microglial cells obtained from newborn rat pups and cocultured with *A. castellanii* were shown to destroy amebae by both phagocytic and lytic processes (Fig. 15). Furthermore, *A. castellanii* and *A. culbertsoni* cocultured with microglial cells induced the production of mRNAs for the cytokines IL-1α, IL-1β, and TNF-α (Fig. 16). These observations that microglia undergo inducible expression of proinflammatory cytokine genes suggest a mode by which *Acanthamoeba* effects neuropathology (271). Studies also have been performed on *Acanthamoeba*-microglia interactions by using highly pathogenic *A. culbertsoni* and weakly pathogenic *A. royreba* amebae. Shin et

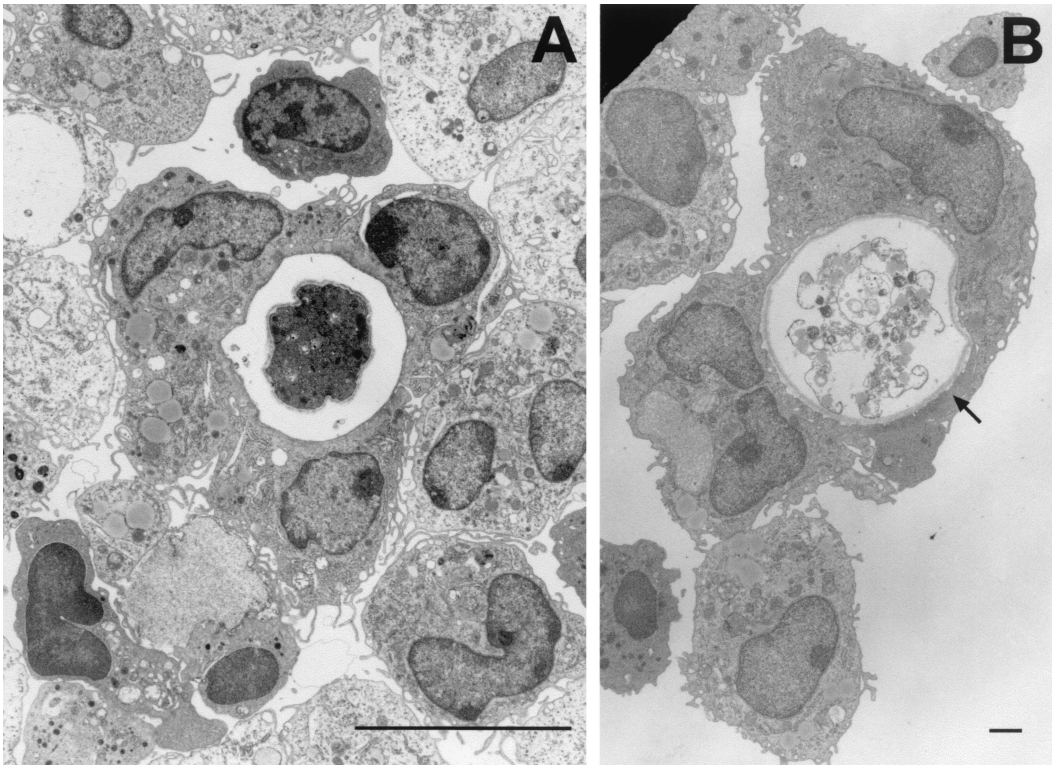

FIG. 14. Transmission electron micrograph illustrating the accumulation of macrophages in focal areas containing amebae in an experimental mouse model of *A. castellanii* infection. Macrophages migrate to sites containing amebae during the later phase of infection. (A) Accumulation of macrophages around an *A. castellanii* cyst (72 h postinfection). (B) Accumulation of macrophages at a site containing amebae (96 h postinfection). Note the presence of an ingested cyst within the macrophage (arrow). Bars, 10 μm (A) and 1 μm (B).

al. reported that microglial cells cocultured with virulent *A. culbertsoni* exhibited cytopathic changes consistent with those described for cells undergoing apoptosis while microglial cells cocultured with weakly pathogenic *A. royreba* did not (411). In view of these observations, it has been postulated that virulent *Acanthamoeba* strains escape the amebicidal activity of macrophages and macrophage-like cells while, in contrast, weakly pathogenic species are targeted by macrophages and are lysed, ingested, and destroyed (271, 272, 411).

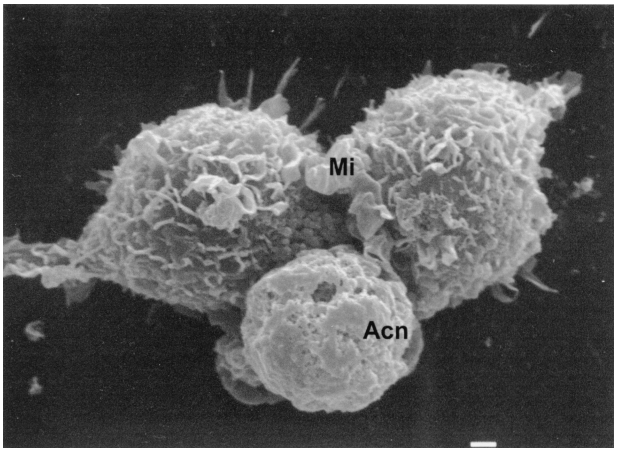

FIG. 15. Scanning electron micrograph of brain microglial cells (Mi) cocultured with *A. castellanii* (Acn). Microglial cells, the resident macrophages in the brain, are capable of injuring *A. castellanii* by cell contact-dependent lysis. Bar, 1 μm.

Few studies have been conducted to investigate the interactions of *Acanthamoeba* with T lymphocytes. Tanaka et al. (439) examined T-cell responses to *Acanthamoeba* antigens in healthy human subjects. T-cell clones from these subjects were established and analyzed for biological and functional activities. Those derived from asymptomatic healthy individuals proliferated in response to *Acanthamoeba* antigens. The T-cell clones were categorized as being Th1 cells involved in delayed-type hypersensitivity reactions in that they produced gamma interferon in response to amebic antigens. Based on their observations, Tanaka et al. (439) suggested that the low incidence of symptomatic disease in humans might be due to acquired protective immunity induced by T-cell responses.

Role of the Immune System in Amebic Keratitis

The cellular immune response to ocular infections with *Acanthamoeba* in a limited number of human patients and in experimental animals has been studied (237, 288). Macrophages and neutrophils are the major inflammatory cell types found in tissues surrounding amebae or cysts. In one set of studies, corneal buttons from two AK patients with chronic inflammation were examined using electron microscopy and immunohistochemistry to identify cell types involved in the inflammatory response (288). The use of monoclonal antibodies to cell surface markers demonstrated that the corneal

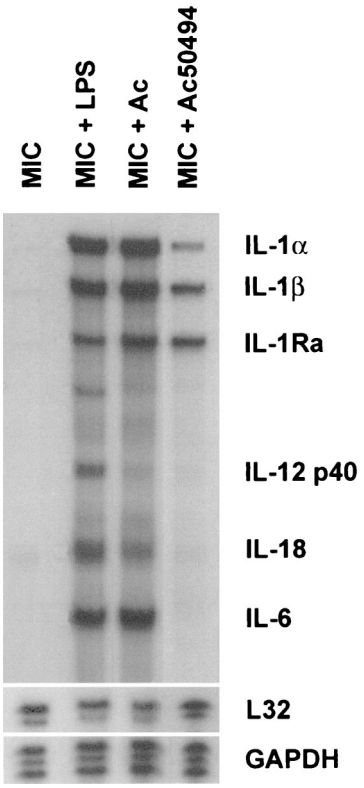

FIG. 16. RNase protection assay illustrating multiple mRNAs elicited by microglia in response to *Acanthamoeba.* Primary microglial cells obtained from brain cerebral cortices of newborn rat were cocultured with *A. castellanii*, *A. culbertsoni*, or bacterial lipopolysaccharide (LPS) for 6 h. The cultures then were harvested with Trizol, and the RNA was subjected to RNase protection assay analysis for assessment of inducible cytokine gene expression. Microglial cells alone (lane MIC), microglia incubated with LPS (lane MIC + LPS), microglia incubated with *A. culbertsoni* (lane MIC + Ac), and microglia incubated with *A. castellanii* (lane MIC + Ac50494) are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

stroma contained polymorphonuclear leukocytes and macrophages with few to no stromal lymphocytes. Despite evidence of a vigorous corneal infiltration by neutrophils and a measurable humoral response, infection in these patients proceeded to corneal opacity or perforation. Based on these observations, Mathers et al. (288) suggested that *Acanthamoeba* masks its antigens from cellular immune responses, thereby suppressing macrophage functions or preventing lymphocyte recruitment.

A variety of animal models including Wistar rats, pigs, mice, and Chinese hamsters have been used for studying immune responses in AK patients after exposure to trophozoites or cysts of human isolates of *A. polyphaga* or *A. castellanii.* An intense inflammatory response consisting of neutrophils and macrophages was shown to develop in Wistar rats following inoculation of *A. polyphaga* directly into the corneal stroma (248, 249). The profile of inflammatory cells changed during progression and clearance of disease. Initially, neutrophils were prominent at sites of infection; this was followed by the accumulation of macrophages, which persisted. A few T cells were present late in infection, but B cells were absent. Protection against ocular infection by *A. castellanii* has also been investigated by using a pig model (6). Pigs immunized with a crude aqueous extract of *Acanthamoeba* intramuscularly, subconjunctivally, or by both routes were exposed to *Acanthamoeba*-laden "soft contact lenses" made of dialysis tubing. Intramuscular injection of ameba extracts failed to protect against AK after *Acanthamoeba* challenge, even though high IgG titers and lymphocyte blastogenic responses developed. Furthermore, previous ocular infection did not protect from reinfection. In contrast, 50% of pigs immunized with amebic extract subconjunctivally and 100% of pigs immunized with both a subconjunctival and intramuscular injection of amebic extract were protected from infection. However, protection did not correlate with levels of humoral antibody or blastogenic responses by peripheral blood lymphocytes. Van Klink et al. (452) studied the immune response during ocular infection with *Acanthamoeba* in Chinese hamsters. Corneal infection with *A. castellanii* failed to stimulate a delayed-type hypersensitivity reaction or serum IgG against *Acanthamoeba* antigens. Subsequent intramuscular immunization with amebic antigens elicited a vigorous delayed-type hypersensitivity and IgG response, indicating that tolerance to amebic antigens had not occurred. The inability to induce a cell-mediated or humoral response during corneal infection was attributed to the absence of resident antigen-presenting cells in the central cornea (452).

Collectively, animal studies indicate that macrophages play a prominent role in providing protection against *Acanthamoeba* ocular infections. Depletion of macrophages with the drug dichloromethylene diphosphonate exacerbated severity of infection in the Chinese hamster and prolonged disease, even though AK is a self- limiting disease in this animal (453). Niederkorn et al. (333) suggested that while macrophages limit the severity and chronicity of corneal disease, they do not inhibit corneal infection. On the other hand, the role of neutrophils in AK remains unclear. McCulley et al. (299) reported that neutrophils are abundant in AK and that the most severe stromal necrosis in *Acanthamoeba* lesions is mediated by proteases released by neutrophils rather than from amebae. However, in the cornea of the Chinese hamster, resolution of disease occurs with production of macrophage inflammatory protein 2 (MIP-2) a chemotactic factor for neutrophils (193). It was demonstrated further that selective inhibition of neutrophil migration in the cornea of Chinese hamsters through exposure to anti-MIP-2, an antibody to neutrophil chemotactic factor, or through elimination of neutrophils by using anti-neutrophil antiserum resulted in prolonged and more severe corneal disease. Furthermore, more rapid resolution of disease occurred when recombinant MIP-2 was injected into the cornea prior to infection.

The role of serum antibodies in AK in humans or experimental animals is not apparent. Walochnik et al. (468) examined sera from 20 individuals, including 2 AK patients, for anti-*Acanthamoeba* IgM, IgG, and IgA by using Western blotting (468). Differences in the immunological reactivity between a highly pathogenic AK isolate and a nonpathogenic isolate were evaluated. All sera were positive for anti-*Acanthamoeba* antibodies, but marked differences in IgA reactivity were observed between sera from infected patients and those from uninfected individuals. In IgA immunoblots, IgA immunoreactivity was significantly higher against the nonpathogenic

strain than against the pathogenic strain, suggesting that pathogenic and nonpathogenic strains elicit distinct IgA antibody profiles. Furthermore, sera from AK patients showed weak immunoreactivity to the pathogenic strain (468). In Chinese hamsters, intramuscular immunization with *Acanthamoeba* antigens elicited a vigorous antibody response but corneal infection proceeded (452). On the other hand, oral immunization with *Acanthamoeba* antigens induced resistance to corneal infection with *Acanthamoeba* in both a Chinese hamster and pig model of AK (252, 255). Protection correlated with production of IgA antibodies. Furthermore, passive transfer of *Acanthamoeba*-specific monoclonal IgA antibodies protected against AK in the Chinese hamster (254). Thus, it appears that a mucosal immune response involving IgA is effective in protecting against *Acanthamoeba* but does not influence the course of infection once it is established (255, 332). Alizadeh et al. (5) have suggested that secretory IgA (sIgA), but not serum immunoglobulin, is important in alleviating AK. In a series of studies, the levels of *Acanthamoeba*-specific serum IgG and tear IgA in 23 amebic keratitis patients and 25 healthy human subjects were assessed by an enzyme-linked immunosorbent assay. Levels of total serum immunoglobulins were shown not to differ significantly between healthy subjects and patients with AK, but levels of ameba-specific tear IgA were significantly lower in patients with AK. Thus, it has been postulated that low levels of tear IgA may be associated with AK. In summary, the cumulative data suggest that both innate and acquired immune responses come into play in AK. IgA may act to prevent the attachment of amebae to corneal cells, while neutrophils and macrophages may migrate to focal areas of infection and injure or destroy amebae.

Virulence Factors

Pathogenic and nonpathogenic strains of *Acanthamoeba* have been isolated from the environment, but the pathogenesis of infection and the biochemical determinants of virulence are poorly understood. Temperature tolerance, growth rate, adherence properties, cytolytic products produced by amebae, and immune evasion mechanisms appear to constitute important factors in pathogenicity. The virulence of pathogenic amebae wanes during continuous culture in axenic medium but can be restored by brain passage in mice (197, 296). It has been proposed that virulence may be related to distinct physiological characteristics of a strain and not to a dependence on environmental conditions (464).

Studies to define pathogenic mechanisms have been conducted more often on strains of *Acanthamoeba* associated with AK rather than on those obtained from the brain. Adherence of trophozoites to cells followed by injury and invasion of tissue are thought to represent important steps in the establishment of infection. Clinical isolates of *A. polyphaga*, *A. castellanii*, and *A. culbertsoni* have been shown to attach to corneal epithelial cells through a process which involves binding to cell surface carbohydrate moieties (318). *A. castellanii* binds to mannose-containing glycoproteins on the corneal epithelium through a 136-kDa mannose-binding protein on the ameba surface (485). The adherence of *Acanthamoeba* to corneal epithelial cells can be inhibited by mannose and methylmannose pyranoside but not by other sugars (318). Glycolipids of corneal epithelium reactive with *Acanthamoeba* may also play a role in the pathogenesis of AK by mediating the adherence of the amebae to the cornea (342). Studies in which monoclonal antibodies were shown to be reactive with different surface membrane epitopes suggest the presence of more than one adhesion molecule on the ameba surface (212). Kennett et al. (212) proposed that an adhesion molecule distinct from the 136-kDa mannose-binding protein, or an adhesion molecule composed of two different subunits, plays a role in attachment of amebae to host cells. Gordon et al. (160), using binding assays, reported that *Acanthamoeba* binds preferentially to collagen IV, laminin, and fibronectin.

Following adherence to cells, invasion and extensive tissue destruction occur in the host. Human epithelial cells, stromal keratocytes, and stromal cell homogenates have been used in vitro as models of AK (432). Damage to cells and tissue is thought to occur by phagocytic processes and by cytotoxic substances released by amebae. Using cultured human epithelial cells, Stopak et al. (432) proposed that pathology associated with AK elicited by *A. polyphaga* occurred when organisms became bound to the epithelial surface and fed directly on cells. Such "direct feeding" resulted in disruption of epithelial layers and invasion of amebae into the stromal layers. Studies with corneal epithelial cells indicated that *Acanthamoeba* can also cause direct target cell cytolysis (18, 247, 432). Electron microscopy studies of human corneal buttons exposed to *Acanthamoeba* indicated that entry into the cornea apparently involved a sequential process in which trophozoite adherence was followed by penetration. This process appeared to involve both secretion of lytic enzymes and phagocytosis (317). Consistent with these observations, Alfieri et al. (4) indicated that enzymes secreted by *Acanthamoeba* may play a role in contact-independent injury to cells and facilitate the invasion of host tissue. Hadas and Mazur (165) compared serine and cysteine proteinases of pathogenic and nonpathogenic species of *Acanthamoeba.* Their studies suggested that cysteine proteinases could serve as markers for pathogenicity since larger amounts were detected in pathogenic strains than in nonpathogenic species of *Acanthamoeba.* Mitro et al. (311) examined excretory and secretory products of a corneal isolate of *A. polyphaga* ATCC 30461 for protease activity. Partial characterization of the products demonstrated that *A. polyphaga* trophozoites secrete multiple proteinases. These included serine proteinases, cysteine proteinases, and metalloproteinases which are able to degrade type I collagen, the main component of the corneal stromal matrix. In addition, Cho et al. (81) purified and characterized a 42-kDa extracellular serine protease which degrades collagen and rabbit corneal extract. The purified proteinase also exerted cytopathic effects (CPE) on corneal epithelial cells and fibroblasts. Secretion of serine proteinases which degrade a broad spectrum of extracellular matrix proteins and serum proteins has been proposed as correlating with invasive properties of *Acanthamoeba* (451).

Ferrante and Bates (137) and He et al. (177) reported that acanthamoebae elaborate elastase, which degrades connective tissue proteins and causes damage to target cells. High levels of elastase activity were found in *A. culbertsoni* and were implicated as being linked to tissue destruction (137). Collagenolytic enzymes which degrade collagen in vitro were studied using *A.*

castellanii ATCC 30868, a strain isolated originally from a human cornea (177). Intrastromal injections of conditioned medium obtained from *A. castellanii* cultures containing collagenase produced corneal lesions clinically similar to those found in biopsy specimens from human patients diagnosed with AK. Van Klink et al. (451) compared the fibrinolytic activity and collagenolytic activity of two strains of *A. castellanii*, one a soil isolate and the other an eye isolate. Fibrinolytic activity was found in the ocular isolate but not in the soil isolate, whereas collagenolytic activity was found in both the soil and ocular isolates. Thus, production of fibrinolytic enzymes and collagenase, or a combination of the two enzymes, by *Acanthamoeba* may correlate with the induction of corneal pathology (451). Plasminogen activator, a 40-kDa serine proteinase, also may play an important role in pathogenesis of AK (310). Collectively, these studies suggest that the pathogenic potential of *A. castellanii* to cause keratitis may be related to its capacity to bind to corneal epithelium, elaborate plasminogen activator, and produce cytopathology on the corneal epithelium (310, 451). Furthermore, proteinases produced by *Acanthamoeba* not only may play a role in pathogenesis of disease and cytopathogenicity of cultured cells but also may serve as markers for differentiating pathogenic and nonpathogenic amebae.

In addition, phospholipase activity is higher in virulent *Acanthamoeba* strains and species than in avirulent amebae (102, 130, 309, 455, 456, 459). For example, phospholipases have been identified in the media of virulent *A. culbertsoni.* Cursons et al. (102) postulated that secreted phospholipases were responsible for extensive demyelination of brain issue. Studies of mice infected with *A. culbertsoni* demonstrated that phospholipase A and sphingomyelinase were elaborated in brain during infection and caused the degradation of phospholipids with an accumulation of free fatty acids and lipid peroxides (244).

Cytopathogenicity of *Acanthamoeba* spp.

The first report that *Acanthamoeba* caused CPE for cells in culture was published in 1957, when these protozoa were found as tissue culture contaminants (202). Since then, *Acanthamoeba* strains have been assessed for CPE by using various mammalian cell lines. Studies have been conducted on biochemical parameters, adherence properties, pathogenic and cytopathogenic mechanisms, and antiamebic drug efficacy. Both contact-dependent and contact-independent mechanisms of target cell injury have been reported (253, 271, 351). Cao et al. (66) suggested that lectin-mediated adherence to host cells was a prerequisite for ameba-induced cytolysis of host cells. However, it also has been reported that cell-free medium containing soluble factors released from trophozoites can cause injury to host cells (253). Leher et al. (253) indicated that cell-free medium obtained from mannose-treated *Acanthamoeba* induced the lysis of corneal epithelial cells. In addition, treatment of the medium containing ameba soluble factors with the serine protease inhibitor phenylmethylsulfonyl fluoride was reported to inhibit most but not all of the cytolytic activity. On the other hand, mannose blocks the binding of trophozoites to cells and inhibits the phagocytosis of red blood cells and yeast (9, 54).

Comparative assessments of the ability of human or environmental isolates of *Acanthamoeba* spp. to destroy mammalian cells have been undertaken (18, 242, 247, 410). In a study of the CPE of an AK isolate of *A. polyphaga* and an environmental isolate of *A. castellanii* using confluent monolayers of human and rabbit corneal cells as targets, total destruction of target cell monolayers was observed (247). The CPE was dependent on the incubation time and on the effector/target ratio of amebae to corneal cells. Badenoch et al. (18) assessed the virulence of strains of *Acanthamoeba* derived from the cornea, the nasal mucosa, or the environment. Isolates were screened for virulence by using a rat model of AK and were assessed for CPE on human keratocytes. An 86% correlation was obtained between virulence of trophozoites in vivo and CPE in vitro. However, not all strains which were virulent in rat corneas in vivo produced CPE on keratocytes in vitro. Eight of 13 corneal isolates, 2 of 9 environmental isolates, and 1 of 6 nasal isolates induced CPE on keratocytes, while 6 of 13 corneal strains, 3 of 9 environmental strains, and 1 of 6 nasal strains induced AK in the rat. Pidherney et al. (353) reported that *A. castellanii* ATCC 30868 isolated originally from human corneal tissue produced CPE on primary pig epithelium, human corneal endothelium, murine neuroblastoma cells, human ocular melanomas, and murine fibrosarcomas. In another study, the ability of *A. castellanii* ATCC 30868 to induce CPE on normal rabbit kidney cells was found to be dependent on the ameba-to-target-cell ratio (335); CPE reportedly did not occur when cocultures were maintained at a low ameba-to-target-cell ratio.

An active phagocytic process in which amebostomes (i.e., food cups) were used to ingest Vero cells was described by Diaz et al. (114) for *A. lenticulata* and an unidentified species of *Acanthamoeba.* Lagmay et al. (242) reported that live amebae, as well as cell-free supernatants prepared from amebae isolated from AK patients and from an environmental isolate, produced CPE on C6 rat glial cells in a dose- and time-dependent manner. These investigators suggested that cytotoxic factors released from amebae during the logarithmic phase of growth accounted for target cell death. Alizadeh et al. (7) reported that *A. castellanii* lysed tumor cells by a process involving apoptosis. Further characterization of the CPE revealed that calcium channels and cytoskeletal elements were involved in the cytopathology of ocular melanoma cells by *A. castellanii* (441). Phagocytosis or trogocytosis of target cells by the ameba was not observed in these studies. In contrast, Pettit et al. (351) demonstrated that trophozoites of four species of *Acanthamoeba* induced the cytolysis of cultured rat B103 nerve cells in addition to ingesting these cells through food cups (Fig. 5). Transmission and scanning electron microscopy studies revealed that *A. culbertsoni*, *A. castellanii*, *A. polyphaga*, and *A. astronyxis* apparently caused cytolysis through a contact-dependent process involving the extension of digipodia from the amebal surface to the target cell membrane (Fig. 17). Membrane blebbing and margination of nuclear chromatin were observed on target cells with which digipodia were in contact, consistent with the induction of apoptosis. In addition, studies of the interaction of *A. castellanii* with human epithelial Wish cells have shown that amebae release ADP into the culture medium, resulting in a biphasic rise in $[Ca]_i$, which culminates in apoptosis (292, 293). We have demonstrated that cell death induced by *Acanthamoeba* spp. also can occur by necrosis (351). Studies indicated that a subset of target neuroblastoma

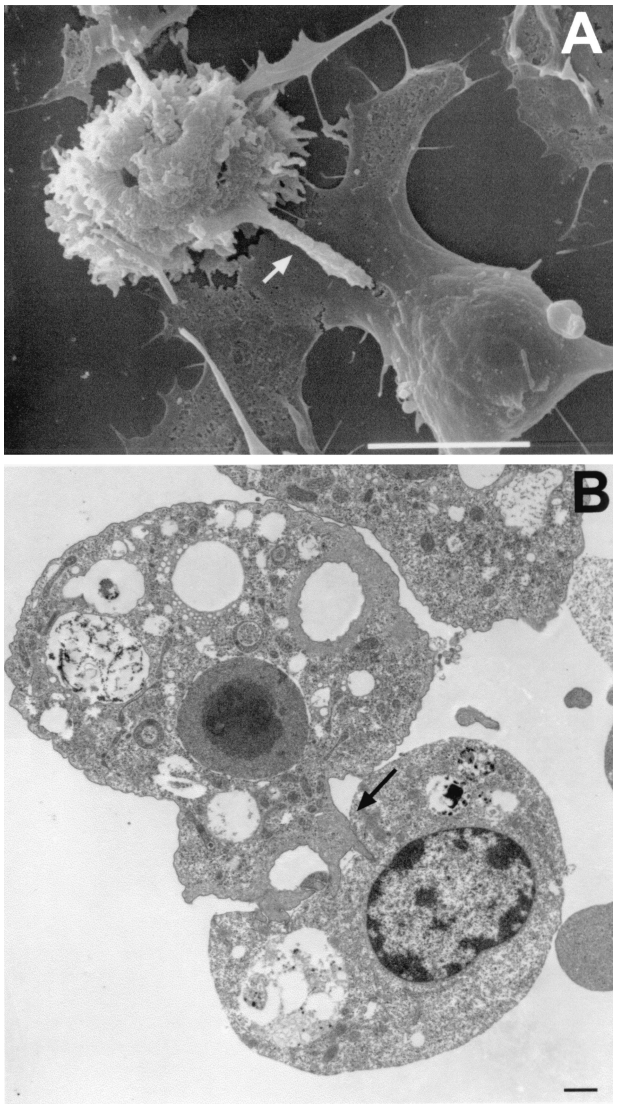

FIG. 17. Scanning (A) and transmission (B) electron micrographs of *Acanthamoeba* cocultured with rat B103 neuroblastoma cells. (A) *A. castellanii* with extended digipodium (arrow) into the cytoplasm of the target B103 cell. (B) *A. culbertsoni* cocultured with B103 cells, demonstrating fingerlike projections extending into the target cell. Cells targeted by digipodia (arrow) eventually die by apoptosis or necrosis. Bars, 10 μm (A) and 1 μm (B).

cells cocultured with *Acanthamoeba* exhibited cell swelling and dissolution of cytoplasmic and cell surface membranes.

Although confluent monolayers of mammalian cells have been used to assess the pathogenicity of isolates, both pathogenic and nonpathogenic strains of *Acanthamoeba* are capable of producing CPE on cell cultures under appropriate conditions (101, 335). However, the data also suggest that not all human or environmental isolates cause symptomatic disease in experimental animals, particularly after the isolates have been maintained in axenic culture. Thus, studies to assess for pathogenicity of *Acanthamoeba* isolates should be complemented with those utilizing in vivo models of infection.

MODELS OF *ACANTHAMOEBA* INFECTIONS

Animal Models of Granulomatous Amebic Encephalitis

Culbertson et al. (99) first established the pathogenicity of *Acanthamoeba* for experimental animals after intracerebral and intraspinal inoculations of amebae which had been isolated as a tissue culture contaminant. These studies indicated that *Acanthamoeba* caused extensive choriomeningitis and destructive encephalomyelitis in monkeys and mice. Intravenous and intranasal inoculations of amebae into mice also resulted in fatal infections. However, intraperitoneal and subcutaneous inoculations did not result in the death of the animals (95, 98). Since these initial studies, the mouse has been the animal model of choice to study CNS infections with *Acanthamoeba.* Several strains of *Acanthamoeba* isolated from the environment have been tested for pathogenicity by using this model. In these experimental animals, CNS infection can be produced by intranasal instillation of amebae. Amebic rhinitis and pneumonitis also develop. Some of the environmental isolates produce an acute fatal encephalitis, while others produce a chronic, nonfatal disease consisting of a subacute and chronic granulomatous response. Lesions present in the nasal mucosa and brain contain trophozoites and cysts. Mice exhibit symptoms of neurological disorders including circling movement, difficulties with equilibrium, hyperactivity, and convulsive seizures (95, 97, 98).

Culbertson et al. (97) reported that *A. castellanii* HN-3 isolated from a human nasal swab and inoculated intranasally into mice produced nasal lesions and then demonstrated passage along nerve fibers and invasion of the olfactory bulb. An intense neutrophil response occurred, which was followed by a mononuclear response of the cerebral hemispheres. In addition, granulomas formed in the brain. Cerva (71, 72) reported that mice inoculated with the Neff strain of *A. castellanii* survived without any sign of illness. However, infection of mice with the A-1 strain of *A. culbertsoni* following intranasal inoculation resulted in the death of most of the animals. The mice died within 10 to 13 days postinoculation and exhibited acute pneumonia and CNS lesions. The growth phase of the amebae appeared to be an important factor influencing mortality in mice. The highest mortality rate was observed when amebae harvested at the beginning of the logarithmic phase of growth were used for inoculation. Cerva (71) also reported that continuous cultivation of amebae in culture medium resulted in decreased virulence. Virulence could be restored by use of amebae in the early logarithmic phase of growth.

Studies by Martinez et al. (283) of experimentally induced murine infections with *Acanthamoeba* demonstrated that the earliest signs of infection were respiratory distress, in which difficulty in breathing was noted. Pneumonia was followed by neurological symptoms consisting of head tilt, circling, twirling, and limb paresis. Fatal encephalitis occurred after the appearance of amebic rhinitis and pneumonitis. Trophozoites were observed frequently in the capillaries of the lungs and brains. In addition, in the brain, *Acanthamoeba* produced a chronic granulomatous encephalitis in which both trophozoites and cysts were found. Based on these observations, Martinez et al. (283) suggested that *Acanthamoeba* invasion of the CNS in mice was a secondary outcome of infection and represented a

metastatic spread from the primary focus of infection in the lungs. Intranasal inoculation of *Acanthamoeba* also results in CNS infection due to invasion of amebae through the olfactory nerves to the olfactory bulbs (98, 270).

Experimental murine models of immune suppression also have been developed. Mice treated with corticosteroids to suppress host defenses have been reported to have greater mortalities (274). Animals maintained on the corticosteroid methylprednisolone or the antibiotic tetracycline and then inoculated intranasally with *A. castellanii* showed significantly higher mortalities than did untreated mice. Steroids also impair neutrophil migration into sites of infection as well as phagocytosis of organisms (274). Recently, mouse models of drug abuse also have been used to assess the effects of illicit drugs on *Acanthamoeba* infection. Marciano-Cabral et al. (270) demonstrated that treatment of mice with delta-9-tetrahydrocannabinol (THC), the major psychoactive and immunosuppressive component of marijuana, exacerbated infection with *A. castellanii* or *A. culbertsoni.* Mice treated with THC had higher mortalities from infection than did similarly infected drug-free control animals. Amebae were isolated from the lungs and brains of drug-treated mice at the time of death, suggesting that THC-induced immune suppression contributed to the disseminated disease.

Animal Models of Amebic Keratitis

A number of experimental animals, including both immunocompetent and immunosuppressed hosts, have been exposed to *Acanthamoeba* by using "contact lenses" made of dialysis tubing or by intrastromal injection of amebae (93, 141, 207). Font et al. (141), using a rabbit model, indicated that necrosis and an inflammatory response were induced after administration of subconjunctival or topical steroids followed by intrastromal inoculations of *A. castellanii.* Treatment of animals with steroids did not enhance the severity of AK or the rate of isolation of amebae from cultures inoculated with ocular material. Badenoch et al. (20, 21) reported that suppurative AK could be induced in rats only when amebae were injected directly into the corneal stroma or when the amebae were coinjected with bacteria such as *Corynebacterium.* A model of AK in pigs also has been developed by using "contact lenses" laden with amebae to mimic human exposure. This model is advantageous for studying ocular infections since the anatomic similarities of the pig eye to the human eye allow for clinical correlation (176). However, unlike the human infection with *Acanthamoeba*, which is prolonged, spontaneous resolution of disease in pigs occurred in 8 to 10 weeks. In addition, Van Klink et al. (450) have developed a Chinese hamster model of AK which has been used extensively because clinically and histopathologically the infection closely resembles the acute stages of infection in humans. Using this model, corneal infections were produced when "contact lenses" containing *Acanthamoeba* were applied to abraded corneal surfaces and maintained in place for at least 5 to 7 days. Typical features included neutrophil infiltration, epithelial ulceration, edema, corneal opacity, and neovascularization. Corneal disease was not produced when "contact lenses" containing amebae were applied to intact corneal surfaces (450). Although the Chinese hamster model of AK provides a means of studying the immune response to *Acanthamoeba* infection and testing therapeutic methods to combat infection, the disease in this model is acute and self-limiting. Thus, to date, animal models which elicit the full spectrum of host-pathogen interactions for progressive chronic AK are unavailable. Furthermore, not all animals are susceptible to infection, which complicates the search for an ideal animal model of AK.

ACANTHAMOEBA-BACTERIUM INTERACTIONS

It is becoming increasingly apparent that free-living amebae interact with bacterial species in the environment. The outcome of the interaction of free-living amebae such as *Acanthamoeba* with different bacteria is complex. Amebae have been reported to exhibit different capabilities for binding and internalizing different species of bacteria (358). Bacterium-amebae interactions may lead to the establishment of an endosymbiotic state or, alternatively, to destruction of either the bacterium or the ameba.

Acanthamoeba spp. have been reported to be predators of bacteria and to control the bacterial populations in soil habitats (376). Soil amebae produce bacteriolytic enzymes which enable the amebae to degrade bacterial cell wall components (122, 381, 473). In aquatic environments, *Acanthamoeba* organisms can be found predominantly at the water-air interface, feeding on bacteria which support their growth (359). Some genera of bacteria are "edible," while others are "nonedible" and are not digested, possibly due to the presence of bacterial toxins, toxic pigments, or outer membrane structures (106, 416, 417). Nonpigmented enterobacteriaceae such as *E. coli* K-12 and *Klebsiella aerogenes* serve as better food sources for *Acanthamoeba* than do bacteria such as *Bacillus subtilis* or *Serratia marcescans* (471). Strains of *A. castellanii* isolated from soil and water sites exhibit predatory activity on cyanobacteria in culture (481). The survival and growth of *Acanthamoeba* in the presence of specific bacteria appears to be influenced by the species and density of bacteria. At low densities, gram-negative bacteria support the growth of *Acanthamoeba.* However, at high densities of bacteria to amebae (i.e., >10 to 1), bacteria such as *Pseudomonas* inhibit the growth of *Acanthamoeba* (470). It is not known whether exotoxins produced by bacteria or changes in pH induced by bacterial growth contribute to the death of the amebae.

A number of *Acanthamoeba* species appear to harbor endosymbiotic bacteria which divide and survive within their cytoplasm. Proca-Ciobanu et al. (360) used electron microscopy to describe rod-shaped bacterial endosymbionts in an environmental isolate of *Acanthamoeba.* Hall and Voelz (168) described the growth and reproduction of bacterial endosymbionts in the cytoplasm of *Acanthamoeba* spp. No evidence of a phagosomal or phagolysosomal membrane surrounding bacterial inclusions was observed. Additionally, *Acanthamoeba* organisms isolated from potable water treatment plants have been shown to harbor Archaea-like endocytobiotic organisms (183). The occurrence of nonculturable bacterial endosymbionts in *Acanthamoeba* isolates also has been reported (144, 145). In both clinical and environmental *Acanthamoeba* isolates, chlamydia-like and rickettsial-related organisms have been identified (11, 38, 144, 145, 153, 162, 185, 304). In one study, at least 26% of isolates obtained from human corneal

specimens and 24% of environmental samples contained intracellular endosymbionts. However, attempts to culture the bacteria apart from amebae were unsuccessful (144). Horn et al. (185) studied a novel group of endosymbionts of *Acanthamoeba* which included environmental and clinical isolates. Endosymbionts belonging to the α subclass of *Proteobacteria* closely related to kappa particles of *Paramecium* were identified. Based on 16S rDNA sequence similarities of bacteria, the endosymbionts were assigned tentatively to existing and new genera, *Candidatus Caedibacter acanthamoebae*, *Candidatus Paracaedibacter acanthamoebae*, and *Candidatus Paracaedibacter symbiosus.* Since these bacteria are related to the *Rickettsiaceae*, it was suggested that the interaction of *Acanthamoeba* with these bacteria might be of clinical significance in the transfer of rickettsia in human disease (185). Additionally, chlamydia-like endosymbionts of *Acanthamoeba* have been implicated in respiratory disease (38). In addition to bacterial endosymbionts which stably infect amebae, other bacteria apparently utilize *Acanthamoeba* as their natural host in the environment, wherein they multiply. The bacteria then lyse the amebae in order to disperse.

The role of *Acanthamoeba* spp. as reservoirs or vectors for human pathogens has been examined. Rowbotham studied the interaction of *Acanthamoeba* with *Legionella pneumophila*, the causative agent of atypical pneumonia in humans (384). Several strains of *L. pneumophila*, normally intracellular pathogens of alveolar macrophages, were found to infect *Acanthamoeba*, indicating that free-living amebae could serve as natural hosts of *Legionella.* Following phagocytosis by *Acanthamoeba*, *Legionella* organisms were observed to "escape" into the cytoplasm rather than being sequestered into lysosomal structures and being degraded by hydrolytic enzymes. Following multiplication, the *Legionella* organisms lysed the amebae and were released into the surrounding environment. Based on these observations, Rowbotham (384) suggested that vesicles filled with *Legionella* or amebae filled with bacteria, rather than free bacteria, were the source of legionellosis. Consistent with the postulate of an interaction between *Acanthamoeba* and *Legionella* is the observation that *Legionella* and amebae have been isolated from the same aquatic environments (22, 52, 140, 239). Indeed, amebae isolated directly from river water or soil have been shown to contain *Legionella* spp. (169, 170, 331). Studies have demonstrated that the temperature at which *Legionella* and the amebae interact is important. *Legionella*-induced lysis of host *Acanthamoeba* cells has been observed to occur at an ambient temperature of 37°C. However, at lower temperatures such as 20°C, a converse interaction occurs in that the amebae phagocytize and digest *Legionella* (13).

Since the initial observations of Rowbotham, the interaction of *Legionella* with free-living amebae has been widely studied (Fig. 18). Uptake of *Legionella* can occur through "coiling" phagocytosis. Bozue and Johnson (48) indicated that following uptake, inhibition of lysosomal fusion with phagosomes containing *Legionella* occurs within *A. castellanii.* Subsequently, intracellular multiplication of *Legionella* and killing of host macrophages and amebae have been described (1, 2, 166, 229, 240, 321). However, while *Legionella* induces apoptotic cell death in macrophages, it reportedly does not do so in *A. castellanii.* Gao and Kwaik (148), using *L. pneumophila* and *A. polyphaga*, confirmed that *L. pneumophila* does not kill amebae by apoptosis but, rather, does so by necrosis. Necrotic cell death induced by *L. pneumophila* and subsequent release of these bacteria from the amebae apparently is mediated by the pore-forming activity of the bacteria. Mutants defective in pore-forming activity fail to exit *Acanthamoeba.*

The mechanisms for recognition, entry, and intracellular proliferation of bacteria in amebae and in mammalian cells may be similar (48, 85, 187, 188). Adaptation of such bacteria to both mammalian cells and amebae suggests an interaction which may confer specified functional attributes to the bacteria. Survival and intracellular growth of bacteria in amebae may prime bacteria for intracellular growth in mammalian cells (24). Cirillo et al. (86) suggested that survival of *Legionella* strains from intracellular digestion in *Acanthamoeba* preadapted the bacteria for invasion of human and animal host cells. Recent studies have shown that the interaction of bacteria with amebae may result in changes in the morphology and physiology of the bacteria. For example, *L. pneumophila* cells grown in *Acanthamoeba* were reported to be smaller than when cultured in vitro, to display different surface properties, and to exhibit enhanced motility (25, 27, 28, 226). In addition, de novo synthesis of select *L. pneumophila* antigens has been demonstrated in bacteria grown in amebae (436). Also, bacteria grown in amebae are resistant to chemical disinfectants, and vesicles containing *Legionella* released from amebae are highly resistant to biocides (25, 33). Furthermore, it has been reported that growth in *A. castellanii* enhances the capacity of *Legionella* to invade macrophages and increases the intracellular replication of the bacteria (84, 85, 312, 328). *Mycobacterium avium* grown in *Acanthamoeba* also demonstrated enhanced entry and intracellular replication in macrophages (87).

The interaction of *Legionella* with free-living amebae raises intriguing possibilities for a functional relevance regarding human disease. For example, *Legionella*-like ameba pathogens (LLAP) which may represent a source of respiratory disease in humans have been identified. These pathogens were first described in 1956 by Drozanski (121) and later named *Sarcobium lyticum. S. lyticum* is an obligate intracellular parasite of small free-living amebae isolated from soil and water and has been shown to enter amebae by phagocytosis, to divide in the cytoplasm, and to result eventually in lysis of the amebae (123). Comparative sequence analysis of the 16S rRNA gene of *Sarcobium* with that of related bacteria revealed the closest relationship to *Legionella* species (147, 423). A number of nonculturable, gram-negative bacilli which multiply intracellularly within amebae have since been isolated and named LLAP 1 through 12 (3, 386). Analysis of the rRNA of the LLAPs has shown that these organisms are related closely to the *S. lyticum* described in 1956 by Drozanski (147). Since the LLAPs do not grow on conventional artificial media, amebae have been used for isolation and propagation of the organisms. One LLAP was isolated originally by amebal enrichment of a sputum specimen from a patient with pneumonia. The patient demonstrated a fourfold rise in antibody titer to the bacteria from "infected" amebae (3, 386). Thus, while the role of LLAPs as human pathogens remains unresolved, serum specimens from patients with pneumonia tested for LLAPs suggest that the organisms play a role in community-acquired pneumonia (275).

Ameba-bacterium interactions may extend to bacteria be-

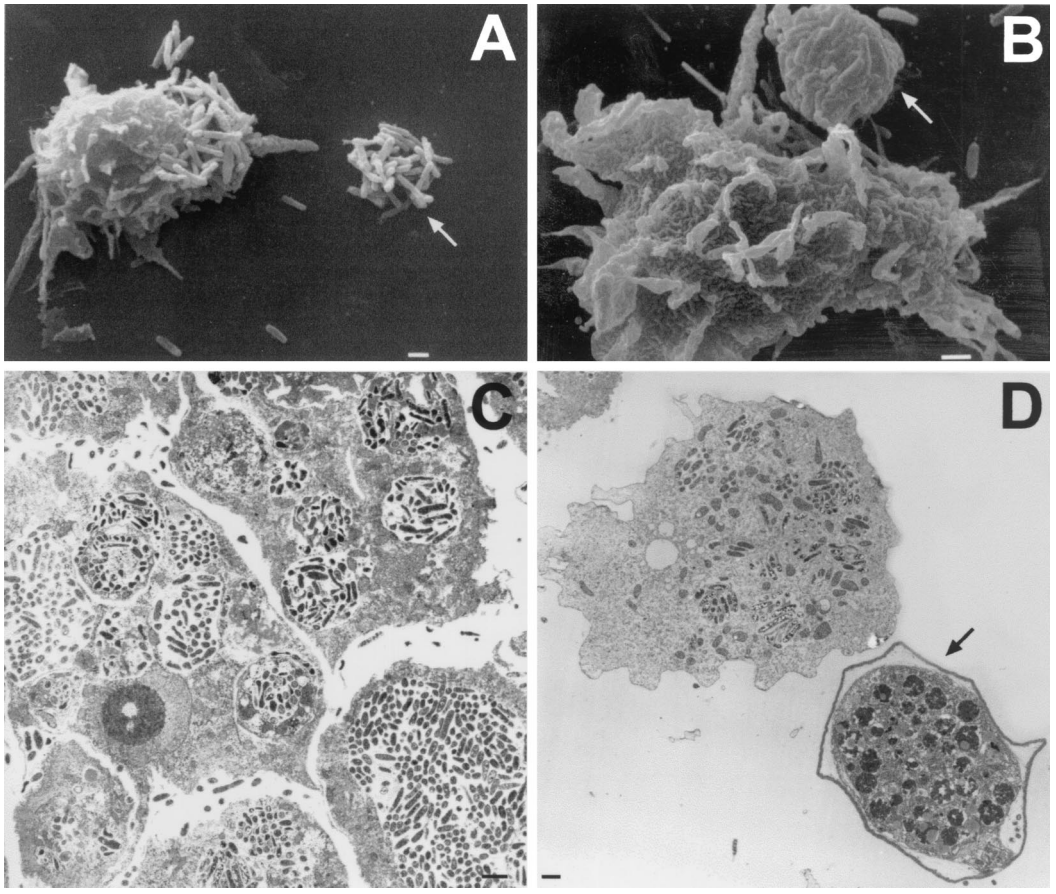

FIG. 18. Scanning (A and B) and transmission (C and D) electron micrographs of co-cultures of *A. astronyxis* and *L. pneumophila.* (A) Trophozoite of *A. astronyxis* extending a fingerlike projection toward a "clump" of *Legionella* (arrow). (B) Trophozoite of *A. astronyxis* infected with *L. pneumophila* and in the apparent process of expelling a vesicle filled with bacteria (arrow). (C) *A. astronyxis* trophozoites harboring numerous *L. pneumophila* cells in cytoplasmic vesicles following coculture in vitro. (D) Cyst of *A. astronyxis* containing *L. pneumophila* organisms (arrow). Bars, 1 μm.

yond *Legionella* and *Legionella*-like species. *Acanthamoeba* organisms recovered from environmental or clinical specimens have been shown to harbor bacterial species including *Candidatus Parachlamydia acanthamoebae* a member of the *Chlamydiaceae*, *Candidatus Odyssella thessalonicensis*, *Rickettsia* spp., *Pseudomonas aeruginosa*, and *Comamonas acidovoran* (11, 37, 144, 302, 303, 464). Laboratory studies using ameba-bacterium microcosms have shown that a number of clinically relevant bacteria such as *Mycobacterium avium*, *M. leprae*, *Burkholderia cepacia*, *B. pseudomallei*, *Simkania negevensis*, *E. coli* O157:H7, *Helicobacter pylori*, *Chlamydia pnuemoniae*, and at least three species of *Listeria* survive and multiply within *Acanthamoeba* (26, 132, 200, 211, 238, 246, 263, 264, 427, 480). Thom et al. (443) showed that *Vibrio cholerae* strains multiply and survive in *Acanthamoeba.* La Scola and Raoult (250a) demonstrated that under experimental conditions, *Coxiella burnetti* is capable of infecting *A. castellanii*, within which it undergoes differentiation into spore-like forms. Gaze et al. (155) reported that in cocultures of *A. polyphaga* and *Salmonella enterica* serovar Typhimurium, invasion of large contractile vacuoles by bacteria occurred. Barker et al. (26) reported that lysed *A. polyphaga* could serve as a substrate for pathogenic *E. coli* O157:H7, the causative agent of hemolytic-uremic syndrome. We have determined that *Helicobacter pylori* multiplies within *Acanthamoeba* and is released in vesicles (Fig. 19). Additionally, *Pseudomonas aeruginosa* was observed in vacuoles when cocultured with *A. polyphaga* under laboratory conditions. Encystment of *A. polyphaga* occurred after several days of cocultivation, with *Pseudomonas* remaining within the cyst compartment (Fig. 20). Steenbergen et al. (424) indicated that the encapsulated yeast *Cryptococcus neoformans* also replicates in *Acanthamoeba* and is not degraded by these amebae. Thus, a variety of bacteria, as well as one species of yeast, are able to survive and/or proliferate within *Acanthamoeba.*

The intracellular growth of bacteria in *Acanthamoeba* has been associated with enhanced survival of bacteria in the environment, increased resistance of bacteria to biocides, and increased bacterial virulence. Intracellular survival within the amebae has been postulated as a mode by which bacteria survive in substrate-limiting environmental ecosystems (227, 240). Intracellular growth of bacteria in amebae apparently also affects the resistance of bacteria to antibiotics (28). For example, *L. pneumophila* grown in *A. polyphaga* and *M. avium* grown in *A. castellanii* were reported to be protected from antimicrobials. Survival studies indicated that *L. pneumophila* isolates grown in *Acanthamoeba*, and tested after release from

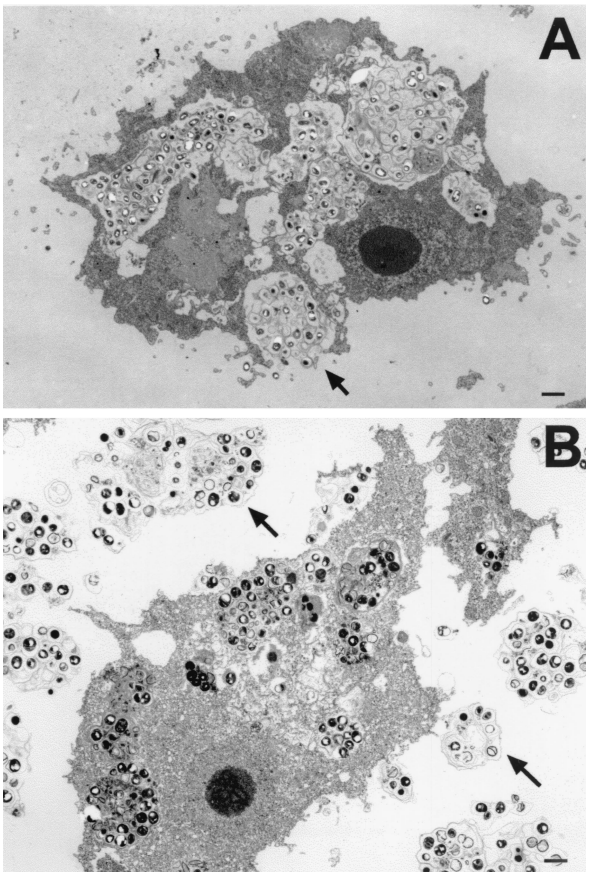

FIG. 19. Electron micrographs of cocultures of *A. astronyxis* and *Helicobacter pylori.* (A) Trophozoite replete with bacteria and in the apparent process of releasing (arrow) a "bolus" of bacteria (96 h coincubation in vitro). (B) Trophozoite containing numerous intracellular vesicles replete with bacteria and surrounded by bacterium-filled vesicles which apparently have been released from the ameba (96 h coincubation in vitro). Bars, 1 μm.

these host cells, were approximately 1,000-fold more resistant to rifampin or ciprofloxacin than were *L. pneumophila* cells grown exclusively in vitro (27). Miltner and Bermudez (308) reported that no significant activity against *M. avium* was observed with rifabutin, azithromycin, and clarithromycin when these antimicrobials were employed to treat infected *A. castellanii.* In contrast, these antibiotics exhibited significant anti-*M. avium* activity when tested against infected U937 macrophage-like cells. These investigators proposed that growth of *M. avium* in amebae reduced the effectiveness of these antimicrobials. It has also been shown that amebae survive chlorination and apparently protect engulfed bacteria such as *S. enterica* serovar Typhimurium, *Yersinia enterocolitica*, *Shigella sonnei*, and *Campylobacter jejuni* from free chlorine (227). Such resistance to chlorination could have health implications, especially for treatment regimens applied to "drinking" water. In addition, *Acanthamoeba* organisms also appear to protect bacteria when the amebae form cysts (385). The double wall of the cyst is resistant to many drugs and chemicals (448). Bacteria which remain in cysts after trophozoites have encysted appear to be protected from chlorine and other disinfectants or biocides (221).

There are accumulating data which suggest a strong potential for *Acanthamoeba* to serve as bacterial reservoirs for human infection. Larkin and Easty (248) demonstrated experimentally that coinfection of rat corneas with *Corynebacterium* and *Acanthamoeba* led to suppurative keratitis whereas infection with either alone did not, suggesting that the presence of bacteria played a role in the establishment of corneal infection. It has also been postulated that a bacterium-related inflammatory response occurs in AK as a result of the release of bacterial endosymbionts from amebae (479). The isolation of *Legionella*, *Pseudomonas* spp., and *Acanthamoeba* from eye wash stations (343) and of *Acanthamoeba* spp. naturally infected with *P. aeruginosa* from a contaminated drinking water system in a hospital (302) further indicates a potential for *Acan-*

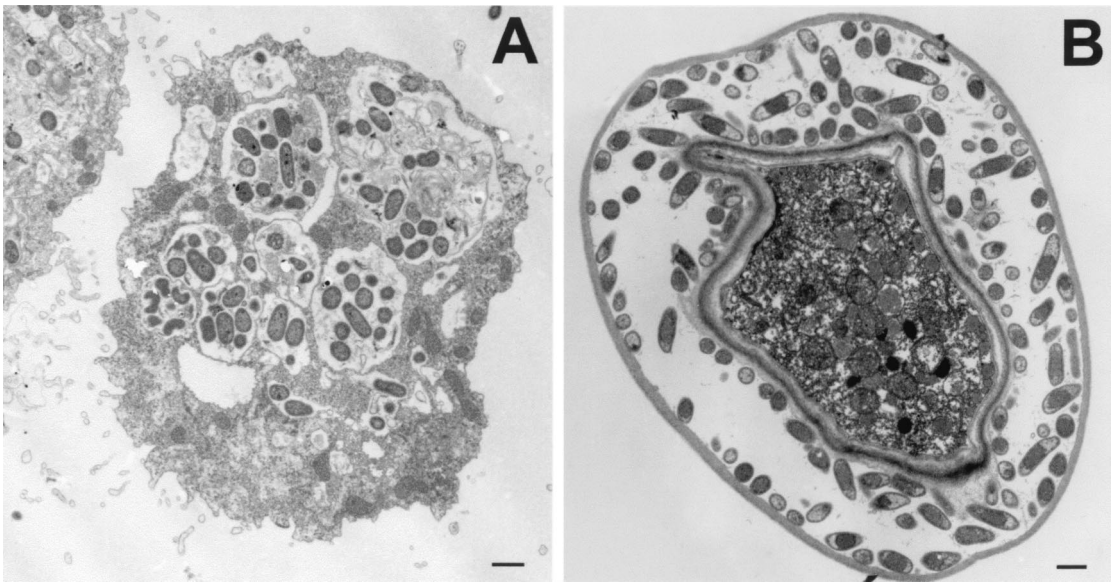

FIG. 20. Transmission electron micrographs of *A. astronyxis* cocultured with *Pseudomonas aeruginosa.* (A) Trophozoite filled with bacteria (72 h of coculture). (B) Cyst of *A. astronyxis* filled with *Pseudomonas* (6 days of coculture). Bars, 1 μm.

thamoeba to serve as reservoirs for human infection. *Acanthamoeba* and bacteria were present on shower heads in bathrooms in a hospital setting in Austria (465). Thermotolerant *Acanthamoeba* organisms were identified which contained an ocular pathogen, *Comamonas acidovorans.* The presence of a *P. aeruginosa* biofilm on the surface of contact lenses has been reported to increase the adsorption of amebic trophozoites to the lens, suggesting an increased opportunity for the development of AK (415). It has also been shown that of several environmental isolates of *Acanthamoeba*, whether obtained through natural or experimental infection, those harboring endosymbiotic bacteria demonstrate CPE on cultured human embryonic fibroblast cells more quickly than do those not containing endosymbionts (146). Induction of CPE was most rapid on monolayers infected with *Acanthamoeba* which included *Chlamydia*-like endosymbionts. In addition, specific antibodies to chlamydia-related organisms, as well as DNA sequences, have been detected in patients with respiratory disease (38). In summary, a body of data indicates that *Acanthamoeba* spp. have the potential to act as cofactors in human infection or as reservoirs for a number of bacterial pathogens.

CONCLUSIONS

Free-living amebae of the genus *Acanthamoeba* are found worldwide and inhabit a variety of water, air, and soil environments. These amebae are the causative agents of GAE, AK, and cutaneous and sinus lesions. While AK may occur in both immunocompetent and immunodeficient individuals, the preponderance of cases of GAE and cutaneous acanthamebiasis have been found in individuals with compromised immune systems, including those with AIDS. Rapid diagnosis of *Acanthmoeba* infections is requisite to the application of successful therapy. Diagnosis and treatment of *Acanthamoeba* infections are difficult due to the rarity of the infections, the lack of familiarity of most clinicians with the disease syndromes, and the limitations of therapeutic options, given the similarity of protozoal physiology to that of other eukaryotic cells, including those of mammals. In vitro culture of amebae and application of specialized staining procedures for tissue sections or cytological preparations are useful in the formulation of a laboratory diagnosis. The use of newer molecular methods to identify *Acanthamoeba* in tissue could provide a more rapid means to diagnose infection. The mechanisms by which *Acanthamoeba* cause disease are unresolved, although it is apparent that multiple modes of action including cell contact-dependent and -independent processes may be involved. Similarly, the role of the immune system in resistance to infection remains unresolved. Recent studies suggest that sIgA, complement, neutrophils, and macrophages may play important roles in resistance to infection. The recognition that *Acanthamoeba* spp. sequester a variety of bacteria with known potential for causing human disease suggests that these amebae serve as reservoirs for bacterial pathogens. Finally, the increase in the reported incidence of *Acanthamoeba* infections may be the result of greater recognition of the disease potential of these organisms. In addition, a number of factors may account for an increased incidence of infection, such as a large number of HIV-infected individuals and more patients undergoing cancer chemotherapy or immunosuppressive therapy for organ transplantation.

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