JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1983, p. 1405–1412
0095-1137/83/121405-08$02.00/0
Copyright © 1983, American Society for Microbiology

Vol. 18, No. 6

Isolation of Two Strains of *Acanthamoeba castellanii* from Human Tissue and Their Pathogenicity and Isoenzyme Profiles

G. S. VISVESVARA,[1]* S. S. MIRRA,[2] F. H. BRANDT,[1] D. M. MOSS,[1] H. M. MATHEWS,[1] AND A. J. MARTINEZ[3]

Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[1]; Veterans Administration Medical Center and Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322[2]; and Presbyterian University Hospital, University of Pittsburgh, Pittsburgh, Pennsylvania 15260[3]

Received 3 June 1983/Accepted 14 September 1983

Two strains of amoebae, one (CDC:0180:1) from the lung tissue of a patient who died of granulomatous amoebic encephalitis and the other (CDC:0179:1) from the debrided tissue of a mandibular autograft, were isolated and identified as *Acanthamoeba castellanii* based on the morphological and immunofluorescent staining characteristics of the trophozoites and cysts. Both strains of amoebae caused cytopathic effects in mammalian cell cultures and destroyed the cell sheet. However, only the CDC:0180:1 strain, on intranasal instillation into mice, produced the disease manifested by ruffled fur and aimless wandering, followed by coma and death within 30 days. The CDC:0180:1 strain also differed consistently from CDC:0179:1 and another nonpathogenic *A. castellanii* strain (ATCC 30,011) in isoenzyme makeup, a dissimilarity which probably reflects its pathogenic potential.

Among the many genera of small, free-living amoebae that inhabit soil and fresh water, members of only two genera, i.e., *Acanthamoeba* and *Naegleria*, cause human disease generally leading to death. Only one species of *Naegleria*, *N. fowleri*, causes a fulminating, rapidly fatal (within 5 to 7 days) disease called primary amoebic meningoencephalitis. Primary amoebic meningoencephalitis afflicts previously healthy children and young adults, usually after fresh water swimming or skiing (14). Several species of *Acanthamoeba*, i.e., *A. castellanii*, *A. culbertsoni*, *A. polyphaga*, and *A. astronyxis*, on the other hand, cause a fatal granulomatous amoebic encephalitis (GAE) with focal necrosis and multinucleated giant cells. GAE has an insidious onset, a prolonged course, and may last for weeks or months. It occurs principally in chronically ill, diabetic, alcoholic, or otherwise debilitated or immunocompromised individuals (8).

A total of 50 cases of primary amoebic meningoencephalitis and 18 cases of GAE have been recorded as having occurred in the United States. Unlike *Naegleria* amoebae, which are often seen in and isolated in culture from the cerebrospinal fluid antemortem and from the brain postmortem, *Acanthamoeba* amoebae have never been isolated in culture from the cerebrospinal fluid or the brain. *A. astronyxis* reported to have been isolated from the cerebrospinal fluid is believed to be a contaminant (2, 7). The patient in that case recovered from the disease after treatment with ampicillin.

We describe in this report the isolation, specific identification, and comparative studies on the pathogenicity and isoenzyme profiles of two strains of *A. castellanii*. One strain was isolated from the lungs of a patient who died of GAE, and the other was isolated from tissue debrided from the site of a mandibular autograft of a 32-year-old woman.

CASE REPORTS

Case 1. A 38-year-old man was admitted to the hospital in January 1980 and evaluated for jaundice. He had received a cadaver kidney transplant 26 months earlier and had developed postoperative pneumonia due to *Legionella micdadei* and cytomegalovirus. On a chest roentgenogram, a nodular lesion on the left lung was noted. A computerized axial tomographic scan of the chest showed multiple noncavitating lung nodules. Hard nodules about 2 cm in diameter, indurated and erythematous, appeared on the skin 4 weeks later. A computerized axial tomographic scan of the head showed multiple areas of low density in the cerebral cortex. A temporal lobe needle biopsy revealed multiple microvascular thrombi. The patient died 42 days after admission. At autopsy, a necrotizing, noncaseating, granulomatous encephalitis was found. Numerous amoebic trophozoites and cysts were seen in the brain and lungs, often in the walls of blood vessels, suggesting vascular dissemination. Amoebae were also found in the adrenal and thyroid glands, lymph nodes, and skin and breast tissue (8).

Case 2. A 32-year-old, mildly obese, prediabetic

woman was admitted to the hospital in January 1979 for treatment of an expansile mass in the right mandibular area. On punch biopsy, an ameloblastoma was diagnosed. The tumor was resected, and an autograft from the right iliac crest was used to fill the defect. Five weeks later, the patient developed an acute suppurative osteomyelitis of the graft and a large fragment of necrotic bone was debrided. Gram-positive cocci, gram-positive and gram-negative bacilli, and trophozoites of *A. castellanii* were cultured from the debrided tissue (1). The patient was discharged 5 days after surgery.

MATERIALS AND METHODS

Protozoology. A small piece (2 by 2 cm) of lung tissue removed at autopsy from the patient in case 1 was frozen and sent to the Centers for Disease Control. It was quickly thawed at 37°C in a small tube containing ca. 1 ml of phosphate-buffered saline (WB saline) (16) and triturated. The resulting thick slurry was then transferred to the center of a 100-mm diameter plastic plate containing a 2-mm layer of 1.5% Difco agar made up in WB saline (nonnutrient agar). The surface of the agar was covered with a suspension of *Escherichia coli* U5-41. The plate was sealed with a strip of parafilm and incubated at 37°C. The plate was microscopically examined daily for several days for the presence of amoebae.

Necrotic tissue removed from the debrided area (from the patient in case 2) was inoculated into thioglycolate broth and transported to the Centers for Disease Control. The broth was chilled and centrifuged at 250 × *g* in a refrigerated centrifuge (International Equipment Co., Needham Heights, Mass.), and the sediment was inoculated onto nonnutrient agar plates and incubated as described above.

Axenic growth. Actively growing amoebae from a 48-h plate culture were scraped from the surface of the plate and suspended in 50-ml of WB saline; the suspension was centrifuged at 250 × *g* for 10 min. The supernatant was aspirated, and the sediment was inoculated into 5 ml of proteose peptone-yeast extract-glucose (PYG) medium containing 400 U of penicillin and 400 μg of streptomycin per ml in a screw-cap test tube (16 by 120 mm) (16). The culture tube was slanted at a 45° angle and incubated at 37°C. The medium was changed twice daily for 2 days and thereafter once daily for 3 more days. After 5 days of incubation, the tube was immersed in an ice-water bath for 6 min, rolled in the palm several times to ensure the detachment of amoebae from the tube wall, and centrifuged as described above. After the removal of the supernatant, the sediment was inoculated into 5 ml of fresh PYG medium without antibiotics. The cultures were tested periodically for bacterial growth by inoculating samples of culture into sterility media such as Bacto stock culture agar, Bacto brain heart infusion, and thioglycolate broth. Sterility tests were made in duplicate and incubated at room temperature and 37°C for 1 week. The cultures were considered axenic if no bacterial growth was observed.

Cell culture. The Vero line of African green monkey kidney cells and MRC-5 human embryonic lung cells were maintained at 37°C in plastic 25-cm^2 tissue culture flasks in Eagle minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Amoebae growing in PYG medium were harvested, washed once in WB saline, counted in a hemacytometer, and suspended in a small amount of WB saline to obtain 50,000 amoebae per ml. A 0.1-ml sample of this suspension, containing 5,000 amoebae, was inoculated onto a 3-day-old monolayer cell culture and incubated at 37°C until the experiment was concluded.

Mouse pathogenicity tests. Outbred 2-week-old Swiss white mice of either sex were lightly anesthetized with ether, and 20 μl of culture medium containing 10,000 amoebae was instilled into the nostril of each mouse. The mice were fed ad libitum on Purina Micro-Mixed Chow and were inspected daily for signs of the disease until they died or were sacrificed. Representative sections of brain, lung, heart, spleen, lymph node, and liver were fixed in 10% buffered Formalin and embedded in paraffin. Sections 4 to 5 μm thick were stained by hematoxylin and eosin, periodic acid-Schiff, Gomori methenamine silver, and acid-fast methods (17).

Indirect immunofluorescent test (IIF). Hyperimmune sera were made in rabbits against *A. castellanii* (ATCC 30,011), *A. polyphaga*, *A. culbertsoni*, *A. astronyxis*, and *A. comandoni* by repeated intravenous inoculations of axenically grown, washed amoebae. The sera were serially diluted twofold in microtitration plates. The diluent used was phosphate-buffered saline, pH 7.6. A 1-drop amount of each dilution of the serum was transferred to individual wells of the 12 wells of Teflon-coated slides to which amoebae had been affixed previously. The IIF test was carried out as previously described (18). Goat anti-rabbit conjugate (Cappel Laboratories, West Chester, Pa.) was used as a dilution of 1:50, and a 1:25,000 dilution of Evans blue was used as the counterstain. The slides were examined with a Leitz Ortholux microscope equipped with a Ploem vertical illuminator, two KP 490 exciter filters, and a K 530 barrier filter (Ernst Leitz, Wetzlar, West Germany) at a ×400 magnification. The intensity of fluorescence was graded on a scale of 0 to 4+; 80% of organisms per field were required to fluoresce at 1+ or greater for the serum to be considered positive. The reciprocal of the highest dilution of serum that reacted at 1+ was defined as the titer of that serum. Sections (6 μm thick) cut from Formalin-fixed, paraffin-embedded brain tissue were deparaffinized, hydrated, and tested by IIF as described by Willaert et al. (19), except that a 1:25,000 dilution of Evans blue was used as a counterstain.

Isoenzyme electrophoresis. The two strains of amoebae under study and *A. castellanii* ATCC 30,011 were grown axenically in PYG medium in 150-cm^2 tissue culture flasks. Amoebae (5 to 6 days old) were harvested and washed three times in WB saline. Amoeba lysates were prepared by four cycles of freezing and thawing and by centrifuging the extracts at 19,000 × *g* in a Sorvall refrigerated centrifuge (model RC 5B; DuPont Instruments, Norwalk, Conn.) for 30 min. The supernatants were subjected to electrophoresis on linear gradient (3 to 23.3% T and 2.6% C) polyacrylamide slab gels for either 2.5 h at 20 mA per gel or 3.5 h at 500 V per gel, and the isoenzyme patterns were visualized by the method of Mathews et al. (11), Sargeaunt et al. (13), and L. E. Munstermann, Ph.D. thesis, University of Notre Dame, Indiana, 1979. The isoenzymes investigated were phosphoglucomutase,

esterase, malic enzyme, malate dehydrogenase, isocitrate dehydrogenase, hexokinase, and superoxide dismutase. Reagents for acrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Calif., and those for enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Protozoology. Agar plates inoculated with materials from case 1 and case 2 were both positive for amoebic growth. On day 2 of inoculation, microscopic examination of the plate surfaces revealed characteristic wave-like track marks as well as numerous amoebic trophozoites. By day 7, the plate was covered with trophozoites and cysts with the characteristic wrinkled outer cyst wall. The amoebae that were recovered from case 1 were designated strain CDC:0180:1, whereas the amoebae cultured from case 2 were designated strain CDC:0179:1.

For large-scale growth, the trophozoites and cysts were gently scraped from the agar surface and inoculated onto fresh agar plates coated with *E. coli*. Amoebae from these plate cultures that were 48 to 70 h old grew luxuriantly after an initial lag when inoculated into PYG medium.

The amoebae of both strains showed similar morphological features; both trophozoites and cysts were uninucleate, and the nucleus was characterized by a large, centrally located, densely staining nucleolus. The trophozoites (Fig. 1) measured 14 to 36 μm with a mean of 20 μm and produced a broad, hyaline lobopodium in the direction of movement. They also exhibited many fine, tapering, thorn-like processes (acanthopodia) which periodically retracted and reformed. A prominent contractile vacuole emptied periodically at the posterior end. The cysts (Fig. 2) measured 10.5 to 22 μm with a mean of 14.3 μm. They were double walled with an outer wrinkled wall and an inner irregularly polygonal or stellate wall. At the point of contact between the outer and inner cyst wall, pores or ostioles were present which were covered with convex-concave opercula. At the time of excystation, the operculum was drawn into the cyst and the trophozoite emerged through the ostiole. The measurements and morphology of the trophozoites and cysts were similar to those described previously for *A. castellanii* (12, 16).

Cell culture. Amoebae belonging to both strains proliferated in cell cultures, formed plaques or cleared areas in the monolayers, and caused cytopathic effects on both types of cell culture. The cytopathic effects consisted principally of nuclear pycnosis and granulation and

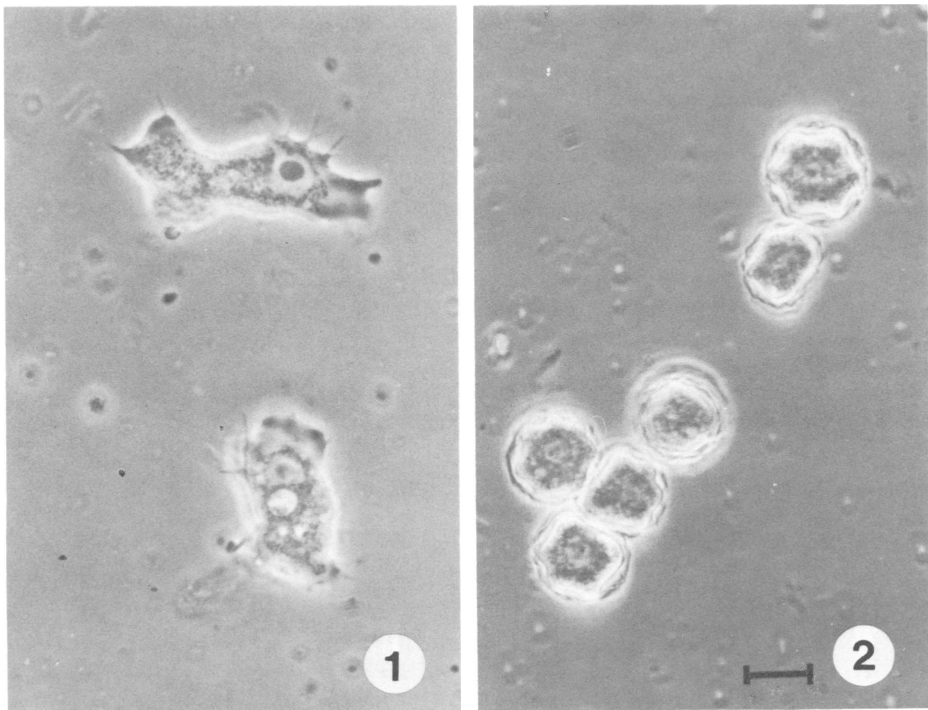


FIG. 1. Trophozoite of *A. castellanii* (CDC:0180:1), phase contrast.
FIG. 2. Cysts of *A. castellanii* (CDC:0179:1), phase contrast. Scale, 10 μm.

shrinkage of cell cytoplasm. The CDC:0180:1 strain, however, produced readily visible plaques within 24 h of inoculation and destroyed the monolayer within 3 days, whereas the CDC:0179:1 strain produced the first signs of cytopathic effects and plaques 48 and 72 h, respectively, after inoculation and destroyed the cell sheet in 5 to 7 days. Both strains of amoeba differentiated into cysts after the cell sheet was destroyed.

Mouse pathogenicity tests. Mice infected with the CDC:0179:1 strain showed no evidence of illness and survived for over 2 months, at which time they were sacrificed. Amoebae were not seen in brain sections nor did they grow on culture. However, mice infected with the CDC:0180:1 strain developed illness manifested by ruffled fur and aimless wandering, followed by coma and death. Table 1 summarizes the number of mice used and their survival time after inoculation with the CDC:0180:1 strain. In this group, brain and lung tissue of dead mice yielded luxuriant cultures on agar plates within 48 h of inoculation of the triturated material.

Pathology of infected mouse tissue. Microscopic examination of infected mouse brains revealed foci of meningoencephalitis involving the temporal and frontal lobes (Fig. 3) as well as the cerebellar hemispheres. The meningeal infiltrate was characterized by an admixture of polymorphonuclear leukocytes and mononuclear cells, i.e., lymphocytes, plasma cells, and histiocytes. The underlying brain parenchyma showed patchy inflammation, often perivascular, with scattered trophozoites and cysts (Fig. 4 and 5). Acute hemorrhagic necrosis was noted in the hippocampus. The olfactory bulbs displayed acute to subacute necrosis with an extensive polymorphous inflammatory infiltrate. There was minimal subependymal inflammation in the caudate nucleus along with degenerating amoebae. The cerebellum showed extensive lesions with cortical necrosis and a diffuse inflammatory infiltrate.

General necropsy findings included a confluent necrotizing pneumonitis (Fig. 6), polymorphonuclear leukocytes, fibrin, and occasional mononuclear cell-filled alveoli and bronchi. Numerous amoebae were present within alveoli and bronchial walls. Perivascular and peribronchial accumulations of plasma cells were also seen. Adjacent abscesses without fibrous walls contained multinucleated giant cells, often with ingested organisms. Focal myocarditis and amoebic trophozoites were observed in the heart, along with inflammation in the epicardial fat. The spleen showed striking replacement with plasma cells. There was congestion of the central veins and sinusoids within the liver without evidence of infection.

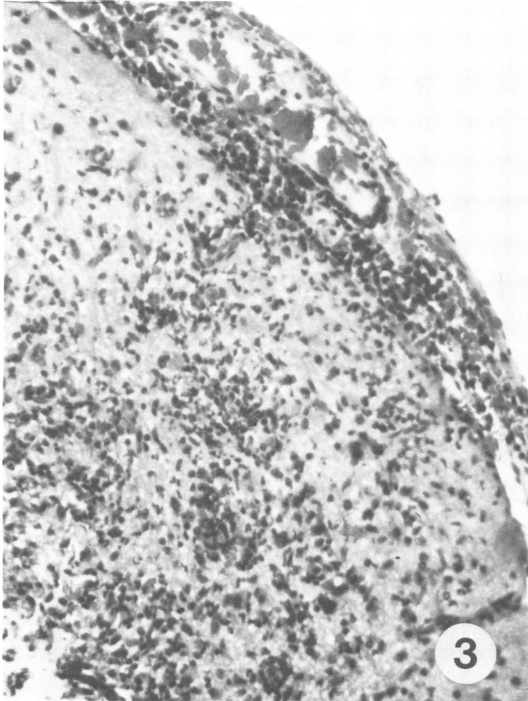
IIF. Both strains (CDC:0179:1 and CDC:0180:1) of amoebae reacted with the anti-*A. castellanii* serum to the same end-point dilution (1:1,024) as the control *A. castellanii* ATCC 30,011 strain (Table 2), strongly suggesting that both strains belong to *A. castellanii*. Amoebae of all three strains fluoresced brightly (4+ reaction) at a 1:64 dilution of the serum. At the 1:1,024 dilution of the serum, however, the fluorescence was confined to the surface membrane with the intensity dropping to 1+. Both strains reacted with anti-*A. polyphaga*, anti-*A. culbertsoni*, anti-*A. astronyxis*, and anti-*A. comandoni* sera (Table 2). Cross-reactivity between *A. castellanii* and *A. polyphaga* was more prominent than that between *A. castellanii* and the three other species. This observation may reflect the morphological similarity of *A. castellanii* to *A. polyphaga*.

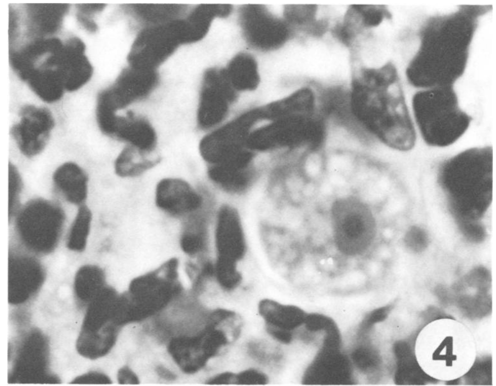
Tissue IIF. Amoebae in the brain sections of the patient in case 1 and from the infected mice reacted with the anti-*A. castellanii* serum at a 1:100 dilution and gave a 2+ reaction (Fig. 7). They did not react, however, when treated with a 1:100 dilution of anti-*A. polyphaga* serum or a 1:20 dilution of the other three sera (Table 3). These observations further point to the fact that the two strains under study are conspecific with *A. castellanii*.

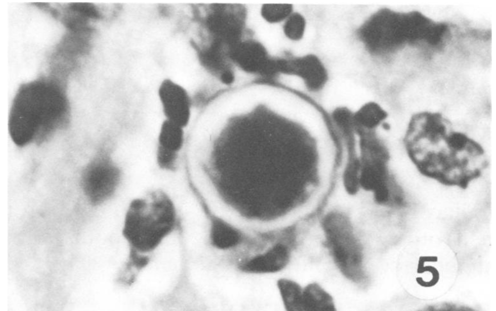
Isoenzyme electrophoresis. The isoenzyme profiles of the three strains studied are depicted in Fig. 8. The CDC:0180:1 strain had distinct isoenzyme profiles and consistently differed from the other two in all of the tested isoenzymes. Differences were observed both in the number of bands produced and the mobilities of the individual bands. Both *A. castellanii* (strain ATCC 30,011) and the CDC:0179:1 strain had

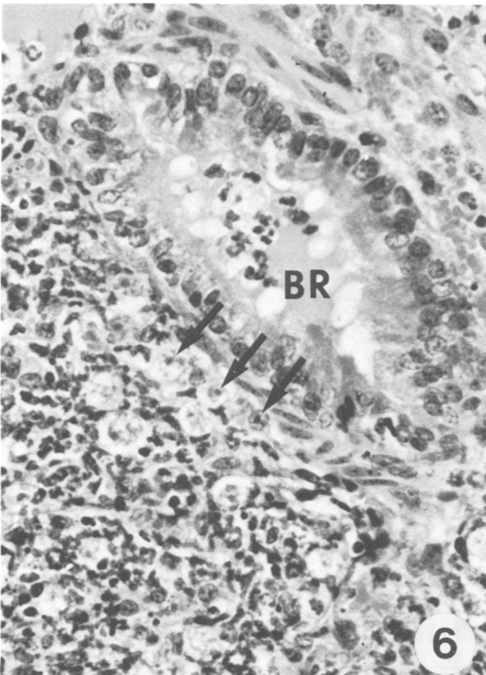
TABLE 1. Infectivity to mice of the CDC:0180:1 strain

Expt no.	No. of mice used	No. of amoebae	No of mice dead	Day of death	% Death
1	8	10,000	8	13, 13, 13, 24, 24, 28, 28, 28	100
2	10	10,000	10	12, 12, 12, 15, 15, 19, 19, 19, 30, 30	100









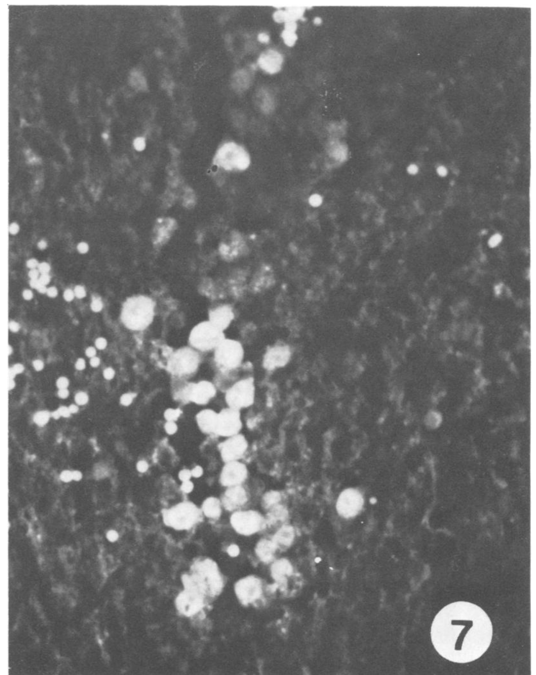


FIG. 3. Section of mouse brain showing a region of necrotizing meningoencephalitis within the cerebral cortex. Stained with hematoxylin and eosin. Original magnification, ×100.

FIG. 4. A trophozoite in the brain tissue of infected mouse. Note the characteristic nuclear morphology. Stained with hematoxylin and eosin. Original magnification, ×1,000.

FIG. 5. A cyst in the brain tissue, with prominent outer wall, hematoxylin, and eosin. Original magnification, ×1,000.

FIG. 6. A section of the lung of the infected mouse; numerous trophozoites (arrows) are seen surrounding a bronchus (BR) in a zone of necrotizing pneumonitis stained with hematoxylin and eosin. Original magnification, ×200.

FIG. 7. Immunofluorescent staining of amoebae in the brain tissue of case 1 after treatment with anti-*A. castellanii* serum. Original magnification, × 100.

TABLE 2. Homologous and heterologous titers of *Acanthamoeba* antisera against selected species and strains of *Acanthamoeba*

Antigens	Titers of antisera				
	A. castellanii (ATCC 30,011)	*A. polyphaga*	*A. culbertsoni* (A-1)	*A. astronyxis*	*A. comandoni*
A. castellanii					
ATCC 30,011	1,024	256	64	<16	16
CDC:0179:1	1,024	256	32	16	16
CDC:0180:1	1,024	256	32	16	16
A. polyphaga	128	1,024	128	16	<16
A. culbertsoni (A-1)	32	32	512	<16	16
A. astronyxis	16	16	<16	1,024	ND
A. comandoni	16	16	<16	ND[a]	512

[a] ND, Not done.

similar hexokinase profiles but differed from each other for other isoenzymes both in the number and mobilities of the bands.

DISCUSSION

Members of the genus *Acanthamoeba* are the commonest amoebae in fresh water and soil. They have often been found as contaminants in bacterial, fungal, and mammalian cell cultures (16).

There is evidence suggesting that *Acanthamoeba* spp., especially the cyst forms, are airborne and may be inhaled. Often incorrectly referred to as "hartmannellid amoebae," they have been isolated from dust in the air and external environment. They have also been isolated from the nasopharynxes of patients with upper respiratory tract symptoms and from healthy individuals (16).

Acanthamoeba spp. have been associated with occasional nonfatal infections, e.g., amoebic keratitis (4, 5), ear infection (6), and pulmonary disease (L. M. Gordeyeva, Abstr. 5th Int. Congr. Protozool. 1977, p. 76). An antibody response to *Acanthamoeba* spp. in people with respiratory problems has also been demonstrated (15), indicating probable previous exposure to the amoeba. Recently, Cleland et al. (3) isolated a strain of *Hartmannella (Acanthamoeba?) rhysodes* from the cerebrospinal fluid of a 30-year-old Nigerian man who made a partial recovery after sulfamethazine therapy. Over a 16-month period, the serum amoeba-immobilization antibody titer of the patient rose from 256 to 1,024.

Despite the wide distribution of the organism in soil, water, and air, fatal *Acanthamoeba* infections have been relatively infrequent. Thirty such cases of GAE ascribed to *Acanthamoeba* spp. have been reported from all over the world; however, the responsible agents were never isolated. Instead, the diagnosis in all these cases was based on the morphology or immunohistology or both of the amoebic trophozoites and cysts.

In this report, we describe the isolation of two strains of *Acanthamoeba* spp., one from the lung tissue of a fatal case of GAE and the other from the mandibular autograft of another patient. Both organisms were identified as *A. castellanii* based on the following findings: the cysts and trophozoites were morphologically identical to those previously described for *A. castellanii*, and both strains reacted with the rabbit anti-*A. castellanii* serum in the IIF test to the same endpoint dilution as the homologous system.

The amoebae in the brain tissue of the patient in case 1 and those in the infected mouse brain derived from the lung of the same patient reacted positively (2+) with a 1:100 dilution of the *A. castellanii* serum in the tissue IIF test. Conversely, the amoebae in the tissue failed to react with antisera against *A. polyphaga*, *A. culbertsoni*, *A. astronyxis*, and *A. comandoni*, at the same dilution. These findings further support the

TABLE 3. Results of indirect immunofluorescence staining of brain sections

Antisera	Dilution	Results
A. castellanii	1:20	4+
	1:50	3+
	1:100	2+
A. polyphaga	1:20	4+
	1:50	2+
	1:100	±
A. culbertsoni	1:20	–
A. astronyxis	1:20	–
A. comandoni	1:20	–

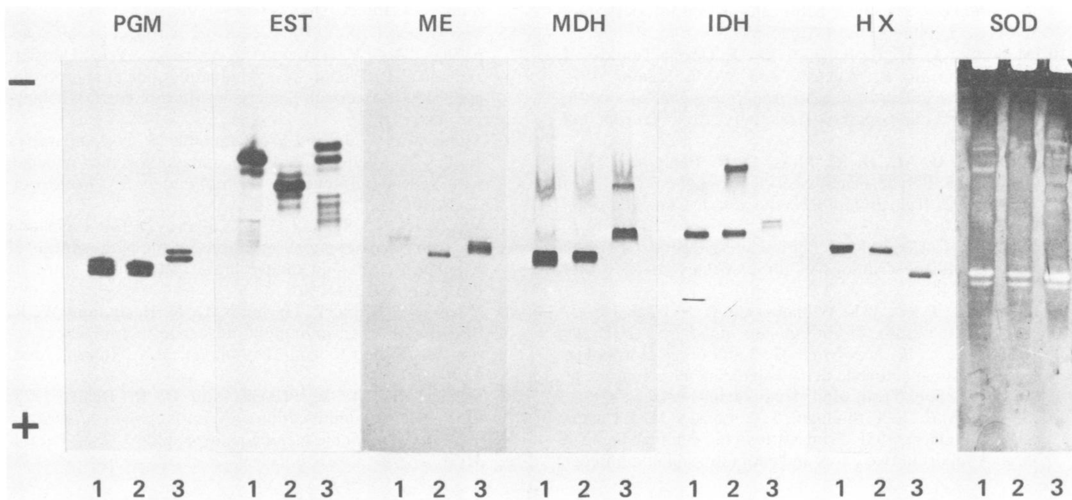


FIG. 8. Isoenzyme profiles of the three strains of *A. castellanii*; (lane 1) strain ATCC 30,011, (lane 2) strain CDC:0179:1, (lane 3) strain CDC:0180:1. PGM, Phosphoglucomutase; EST, esterase; ME, malic enzyme; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; HX, hexokinase; SOD, superoxide dismutase.

identification of the CDC:0180:1 strain as *A. castellanii*. The CDC:0180:1 strain differed consistently from the other two strains of nonpathogenic *A. castellanii* in its isoenzyme profiles. This is probably a reflection of the pathogenicity of the CDC:0180:1 strain.

We also compared the pathological findings in the infected mice with those reported in humans and experimental animals. Naturally occurring *Acanthamoeba* infection in man and laboratory infections in mice usually present as subacute to chronic granulomatous meningoencephalitis with mononuclear cells, i.e., lymphocytes, plasma cells, and histiocytes dominating the picture (7, 9, 10). In the mice infected with the CDC:0180:1 strain of *A. castellanii*, however, a mixed acute granulomatous inflammation was observed.

In contrast to *N. fowleri*, which gain access to the central nervous system via the olfactory neuroepithelium with ensuing necrosis of the olfactory bulbs, *Acanthamoeba* infections reportedly show relative sparing of the olfactory bulbs with a more posterior distribution of the central nervous system lesions (9). However, severe necrosis of the olfactory bulbs, seen in our mice, may be the result of the intranasal route of inoculation. Necrotizing pneumonitis noted in experimental murine *Acanthamoeba* infection prompted Martinez to suggest a pulmonary source for the disseminated infection (9). An extensive confluent necrotizing pneumonitis was the overriding pathological finding in the mice herein inoculated with strain CDC:0180:1.

The plasmocytosis in the mice spleens suggests an immune response to the infection. In fact, an antibody response to *Acanthamoeba* spp. has been shown (19), and cell-mediated immunity has been postulated in the granulomatous reaction of *Acanthamoeba* infection (7).

In summary, we have isolated and identified two strains of *A. castellanii* from two patients. The strain isolated from a fatal case of GAE was used to infect mice who developed meningoencephalitis and pneumonitis.

ACKNOWLEDGMENT

We thank George R. Healy for helpful suggestions and criticisms.

LITERATURE CITED

1. **Borochovitz, D., A. J. Martinez, and G. T. Patterson.** 1979. Osteomyelitis of a bone graft of the mandible with *Acanthamoeba castellanii* infection. Hum. Pathol. **12**:573–576.
2. **Callicott, J. H., C. E. Nelson, M. M. Jones, J. G. dos Santos, J. P. Utz, R. J. Duma, and J. V. Morrison, Jr.** 1968. Meningoencephalitis due to pathogenic free-living amoebae. J. Am. Med. Assoc. **206**:579–582.
3. **Cleland, P. G., R. V. Lawande, G. Onyemelukwe, and H. C. Whittle.** 1982. Chronic amoebic meningoencephalitis. Arch. Neurol. **39**:56–57.
4. **Jones, D. B., G. S. Visvesvara, and N. M. Robinson.** 1975. *Acanthamoeba polyphaga* keratitis and *Acanthamoeba uveitis* associated with fatal meningoencephalitis. Trans. Ophthalmol. Soc. U.K. **95**:221–232.
5. **Key, S. N., III, W. R. Green, E. Willaert, A. R. Stevens, and S. N. Key, Jr.** 1980. Keratitis due to *Acanthamoeba castellanii*. Arch. Ophthalmol. **98**:475–479.
6. **Lengy, J., R. Jakovlzevich, and B. Talis.** 1971. Recovery of a hartmannellid amoeba from a purulent ear discharge. Trop. Dis. Bull. **68**:818.
7. **Martinez, A. J.** 1980. Is *Acanthamoeba* encephalitis an opportunistic infection? Neurology **30**: 567–574.
8. **Martinez, A. J.** 1982. Acanthamoebiasis and immunosuppression. J. Neuropathol. Exp. Neurol. **41**:548–557.
9. **Martinez, A. J., S. M. Markowitz, and R. J. Duma.** 1975. Experimental pneumonitis and encephalitis caused by

Acanthamoeba in mice: pathogenesis and ultrastructural features. J. Infect. Dis. **131**:692–699.

10. **Martinez, A. J., G. Sotelo-Avila, J. Garcia-Tamayo, J. Takano-Moron, E. Willaert, and W. P. Stamm.** 1977. Meningoencephalitis due to *Acanthamoeba* sp.: pathogenesis and clinicopathological study. Acta Neuropathol. **37**:183–191.
11. **Mathews, H. M., D. M. Moss, G. R. Healy, and G. S. Visvesvara.** Polyacrylamide gel electrophoresis (PAGE) of isoenzymes from *Entamoeba* species. J. Clin. Microbiol. **17**:1009–1012.
12. **Page, F. C.** 1967. Re-definition of the genus *Acanthamoeba* with descriptions of three species. J. Protozool. **14**:709–724.
13. **Sargeaunt, P. G., J. E. Williams, and R. A. Neal.** 1980. A comparative study of *Entamoeba histolytica* (NIH:200, HK 9, etc.), "*E. histolytica*-like" and other morphologically identical amoebae using isoenzyme electrophoresis. Trans. R. Soc. Trop. Med. Hyg. **74**:469–474.
14. **Stevens, A. R., S. T. Shulman, T. A. Lansen, M. J. Cichon, and E. Willaert.** 1981. Primary amoebic meningoencephalitis: A report of two cases and antibiotic and immunologic studies. J. Infect. Dis. **143**:193–199.
15. **Visvesvara, G. S.** 1980. Free-living pathogenic amoebae, p. 704–708. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.). Manual of clinical microbiology, 3rd ed., American Society for Microbiology, Washington, D.C.
16. **Visvesvara, G. S., and W. Balamuth.** 1975. Comparative studies on related free-living and pathogenic amoebae with special reference to *Acanthamoeba*. J. Protozool. **22**:245–256.
17. **Visvesvara, G. S., and C. S. Callaway.** 1974. Light and electron microscopic observations on the pathogenesis of *Naegleria fowleri* in mouse brain and tissue culture. J. Protozool. **21**:239–250.
18. **Visvesvara, G. S., P. D. Smith, G. R. Healy, and W. R. Brown.** 1980. An immunofluorescence test to detect serum antibodies to *Giardia lamblia*. Ann. Intern. Med. **93**:802–805.
19. **Willaert, E., A. R. Stevens, and G. R. Healy.** 1978. Retrospective identification of *Acanthamoeba culbertsoni* in a case of amoebic meningoencephalitis. J. Clin. Pathol. **31**:717–720.