Five Cases of Primary Amebic Meningoencephalitis in Mexicali, Mexico: Study of the Isolates

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Five Naegleria strains isolated from patients with primary amebic meningoencephalitis and one strain isolated from the water of an artificial canal were investigated. All strains were pathogenic for mice when instilled intranasally and showed cytopathic effects in vero cell cultures. Their growth characteristics (isolation and subculture at 45°C), serological results, and isoenzyme patterns permitted us to identify the six strains as Naegleria fowleri. This is the first time that Naegleria fowleri has been isolated from patients with primary amebic meningoencephalitis in Mexico.

Naegleria fowleri is a free-living amoeb-flagellate that causes primary amebic meningoencephalitis (PAM) in humans. PAM almost always leads to death after 1 to 2 weeks from the time of acquisition. This ameba has been found throughout the world, mostly in heated aquatic environments, whether artificial or natural (5, 11, 21).

Other thermotolerant species of Naegleria, N. lovaniensis and N. australiensis, have also been isolated from warm water (7). N. australiensis, like N. fowleri, is pathogenic for mice, but the two species are antigenically and biochemically different (2). N. lovaniensis, a thermophilic environmental isolate, is antigenically similar to N. fowleri but is not pathogenic for mice (10). N. lovaniensis and N. australiensis have previously been isolated from thermal waters in Mexico (13). We report the isolation and specific identification of six Naegleria strains, five from patients with PAM that occurred in 1990 in the warm water of Mexicali, Baja California, Mexico, and one from a water sample from an artificial canal in which the victims were swimming before they contracted the disease.

MATERIALS AND METHODS

Ameba strains and isolation methods. All five strains from humans were isolated and were initially cultured on 1.5% nonnutrient agar plates covered with living Escherichia coli NNE by depositing 0.5 ml of premortem cerebrospinal fluid. All five patients were known to have either swam or bathed in an artificial canal in the Mexicali Valley (Table 1). The Mexicali Valley, which is contiguous with the Imperial Valley in southern California, is a desert environment with an intensive agricultural system that depends on an extensive irrigation system originating from the Colorado River. The irrigation system consists of a network of canals and reservoirs whose volumes vary with the irrigation cycle (16). Water samples were collected from five different sites in the canal in which the patients swam or bathed. The temperature and pH of the water samples were also recorded at the time of collection. Briefly, sterile 500-ml glassware was filled with water obtained from the water surface, and the water temperature was measured. The samples were processed on the same day of collection by methods described elsewhere (3). Samples of 50 ml were centrifuged, all but 1 ml of the supernatant was discarded, and the sediment was mixed in the remaining 1 ml of water. This suspension was spread evenly on 1.5% nonnutrient agar covered with living E. coli NNE. The plates were incubated at 45°C in a humidified atmosphere and were examined on the following day for cleared zones (plaques).

Identification of amebae. All plaques were transferred with the supporting agar to a fresh NNE plate. Identification of the genus was done by observation of the vegetative and cystic forms as well as by enflagellation experiments with distilled water at 37°C. The Naegleria isolates were grown axenically by inoculating the ameba growing on agar plates with the bacteria into Cerva’s medium (2% Bacto Caseitone supplemented with 10% bovine serum) (1) and were incubated at 30°C.

Pathogenicity tests. Amebae from axenic cultures were tested for their cytopathic effects on Vero cell cultures. The amebae were counted in a hemacytometer after concentration, washing, and resuspension in phosphate-buffered saline (PBS; pH 7.2). One tenth of a milliliter containing 10⁵ amebae was added in duplicate to the cell culture (1.92 cm² well growth area) immediately after the last medium change. Cultures were incubated at 37°C with 5% CO₂ and were examined daily for a cytopathic effect. Only when complete destruction of the cell monolayer occurred was the cytopathic effect considered positive.

Amebae from the same axenic cultures were tested for

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their virulences in mice. Three-week-old CD-1 strain mice were anesthetized with ether, and two drops of the concentrated culture were instilled intranasally. Mice were observed for symptoms of meningoencephalitis for 3 weeks, and those suffering from PAM were autopsied. Portions of the brain were inoculated onto NNE plates.

**Indirect immunofluorescence antibody technique.** The five strains of amebae from humans (RPE-1, PLC-2, RTW-4, RC-5, and FFS-6) as well as reference strains of *N. fowleri* (HBWS-1, HB-3, and HB-1) and one strain each of *N. lovaniensis* (76/15/250), *N. jadini* (0400), and *N. gruberi* (EG) were grown in large lots in modified Nelson’s medium as described previously (18), with the exception that the concentration of fetal bovine serum was reduced from 10 to 5%. *N. australiensis* ATCC 30958 was grown in TYPH medium (19). All strains except *N. gruberi* EG were grown at 37°C; *N. gruberi* EG was grown at room temperature.

Amebas were washed three times in WB saline (17), counted in a hemacytometer, and suspended in WB saline containing 1% formalin to obtain 10⁶ cells per ml. Aliquots of 10 μl were deposited on each of 12 wells of Teflon-coated slides. The slides were air dried, wrapped in aluminum foil, and stored at −20°C until use.

Monoclonal antibody IV-DI-30 (19) was serially fourfold diluted, beginning at a dilution of 1:16, with PBS (pH 7.6). One drop of each dilution of the monoclonal antibody was placed into the individual wells of the ameba-coated slides, and the slides were incubated at 37°C for 20 min in a humidified chamber. The slides were next washed three times, 10 min per wash, in PBS. The individual wells were covered with a 1:50 dilution of fluorescein-conjugated immunoglobulin G (IgG) fraction of goat anti-mouse immunoglobulins (IgA, IgG, and IgM; lot 17,543; Cappel Laboratories, West Chester, Pa.). Evans blue diluted to 1:25,000 was used as the counterstain. The slides were incubated and washed as described above and were examined with an Olympus fluorescence microscope equipped with epifluorescence.

**Agarose isoelectric focusing.** Isoenzyme patterns were studied as described by De Jongckheere (4). Reference strains and isolates were grown in serum casein glucose yeast extract medium and were harvested at the time of peak growth by centrifugation at 1,500 × g for 10 min. The pellet was suspended in 0.1 ml of 0.25% Triton X-100. The amebic lysates were kept frozen (−20°C) until they were used. Samples of the crude extract (20 μl) were used for each separation of proteins by agarose isoelectric focusing. The zymograms examined were acid phosphatase and propionyl esterase (4).

**Reference strains.** The following reference strains were used: *N. fowleri* European (0.359), American (6488), and Australian (NF66) strains and HBWS-1, HB-3, and HB-1; *N. lovaniensis* 76/15/250 and Ag9/14/5D; *N. jadini* 0400; *N. gruberi* EG; *N. australiensis* ATCC 30958 and PP397; and *N. andersoni* jamiesioni.

### RESULTS

The cases of PAM occurred in August and September 1990 and were diagnosed by finding trophic forms of ameba during direct microscopic observation of samples of cerebrospinal fluid from the patients. The patients’ age, sex, and duration of illness and the strains isolated from the patients are given in Table 1. The clinical and epidemiological findings will be published elsewhere.

All cerebrospinal fluid samples were positive for *Naegleria* species, and amebae were isolated in cultures of cerebro-

### TABLE 1. History of five strains isolated from patients with PAM in Mexicali, Mexico, in 1990

<table>
<thead>
<tr>
<th>Patient no.*</th>
<th>Patient age (yr)</th>
<th>Time of illness before death (days)</th>
<th>Exposure to fresh water</th>
<th>Strain code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>7</td>
<td>Swam 5 days before illness</td>
<td>RPE-1</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5</td>
<td>Swam 5 days before illness</td>
<td>PLC-2</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>5</td>
<td>Swam every day before illness</td>
<td>RTW-4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
<td>Was bathed every day before illness</td>
<td>RC-5</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>3</td>
<td>Swam 9 days before illness</td>
<td>FFS-6</td>
</tr>
</tbody>
</table>

* All patients were males.

spinal fluid from all five patients. However, amebae were cultured from only one of the five water samples. The pH of the water samples ranged from 8.3 to 8.6, and the temperature at noon was between 28 and 32°C.

The results of ameboflagellate transformation, growth temperature, and pathogenicity tests are given in Table 2. Figures 1 and 2 depict the agarose isoelectric focusing isoenzyme patterns for acid phosphatase and propionyl esterase, respectively, of the isolates compared with those of the reference strains. The isoenzyme patterns identified all Mexican isolates as *N. fowleri*.

The immunofluorescence test revealed that the three reference *N. fowleri* strains—HB-1, HB-3, and HBWS-1—and the five Mexican strains isolated from cerebrospinal fluid reacted brightly with the monoclonal antibody at a dilution of 1:1,024, producing an apple green fluorescence. None of the other species of *Naegleria* tested reacted with the monoclonal antibody, which also clearly indicated that the five Mexican strains are similar to strains HB-1, HB-3, and HBWS-1 and correspond to *N. fowleri*.

### DISCUSSION

Only three cases of PAM have so far been reported in Mexico (9, 15, 16). A possible outbreak of PAM was detected in 1989 by an epidemiologic study in Mexicali (unpublished data). As a consequence, an epidemiologic

### TABLE 2. Enflagellation, growth, and pathogenicity results of *Naegleria* species isolated from five patients with PAM and one isolate from canal water

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time of death of mouse no. (days after i.n. inoculation)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>RPE-1</td>
<td>5*</td>
</tr>
<tr>
<td>PLC-2</td>
<td>5*</td>
</tr>
<tr>
<td>RTW-4</td>
<td>6*</td>
</tr>
<tr>
<td>RC-5</td>
<td>6*</td>
</tr>
<tr>
<td>FFS-6</td>
<td>7*</td>
</tr>
<tr>
<td>EJL-SONc</td>
<td>6*</td>
</tr>
</tbody>
</table>

* All strains had a positive enflagellation test result, all strains grew on *E. coli* NNE at 45°C, and all strains exhibited a cytopathic effect at 37°C 24 h after inoculation.

* i.n., intranasal instillation; S, survived; *, brain positive for *Naegleria* species.

* Strain isolated from canal water.
surveillance program was installed in June 1990, and 2 months later, several new cases were detected; this resulted in the isolation of the five strains of amebae from the cerebrospinal fluid of these patients. Isolation of *N. fowleri* from the incriminated environmental sources following proven cases of PAM has been beset with difficulties (20). Although cases of PAM occurred in Czechoslovakia during the 1960s, it was not until 1978 that Cerva and colleagues (8) were able to isolate *N. fowleri* from an epidemiologically incriminated indoor swimming pool. During the summer of 1971, Nelson (12) isolated *N. fowleri* from a water sample taken from a pond in Richmond, Va., where a victim of PAM swam in 1967. In Belgium, De Jonckheere and colleagues (6) isolated *N. fowleri* in 1975 from a thermally polluted canal associated with a fatal case of PAM in 1973. It is important to point out here that this is the first time in Mexico that *N. fowleri* was isolated and grown in culture from an environmental sample as well as from five humans with PAM. Although there is one report of the isolation of *N. lovaniensis* from the cerebrospinal fluid of a child with Arnold-Chiari syndrome (14), *N. fowleri* has not been isolated so far. Previous cases of PAM in Mexico were identified by immunological methods on tissue (9, 16). The indirect immunofluorescence antibody technique and isoelectric focusing identified the isolates as *N. fowleri*, while the pathogenicity test in mice confirmed their virulence. It is therefore important to monitor the quality of the water that runs in the irrigation channels during different seasons to assess the presence as well as the distribution of *N. fowleri* over a period of time so that preventive measures can be instituted.

Since Mexicali Valley is characterized by a desert environment, high temperatures are prevalent, especially during the summer months. Since no recreational facilities are available in the valley, swimming and wading in the canals are pastimes for the people, especially the youth, who live in the valley. Although *N. fowleri* is known to be susceptible to chlorine, it is not realistic to chlorinate the canal water. It is therefore expected that more cases of PAM will occur in the years to come. In view of this, it is advisable to declare this area a zone in which PAM is endemic, and protective measures such as those developed in southern Australia (11) should be instituted.

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**REFERENCES**

