Temperature Tolerance of Pathogenic and Nonpathogenic Free-Living Amoebas

Abstract. *Within tested strains of the genera* Naegleria *and* Acanthamoeba *the ability to grow at high temperatures seems directly related to virulence, with nonvirulent strains unable to grow at normal or elevated body temperatures. Outside these genera, nonvirulent* Hartmannella *and* Tetramitus *do grow at elevated temperatures, which suggests a barrier to pathogenicity other than temperature sensitivity. The high optimal temperature of pathogenic* Naegleria *apparently explains previous difficulty in obtaining isolates from the aquatic environment.*

Primary amoebic meningoencephalitis is a fatal human disease in which freshwater amoebas invade the brain. Human fatalities were first reported (*1*) as due to *Acanthamoeba* (often called *Hartmannella*) (*2*), which is known to cause the disease in experimental animals (*2, 3*). Since then, amoebas cultured from patients (*3, 4*) have all proved to be amoeboflagellates, of a species now named *Naegleria fowleri* (*5*). Despite much interest, only recently and only twice has virulent *Naegleria* been isolated from swimming areas linked to the disease (*6, 7*).

Species of *Naegleria* and *Acanthamoeba* grow in almost all bodies of fresh water. The pathogenic potential of many strains has been tested in mice by nasal introduction of amoebas (*2–4, 8, 9*). Virulent strains from human patients rapidly kill mice (*3, 4, 8*). A test of the idea that temperature tolerance is related to virulence (*10*) is reported here.

The temperature-gradient block (*11*) used in the experiments had 20 steps, from 29° to 48°C, and 200 holes. Thermometers inserted through the insulated lid were monitored, and varied no more than 0.1°C.

Nutritional requirements in axenic culture differ for different amoebas, so all amoebas in this study were fed bacteria. Temperature tolerances in culture with bacteria may differ from those in a host animal, but the conditions are uniform and close to optimal for bactivorous amoebas.

A plug of agar with amoebas was placed on a slant (*12*). After a population of amoebas was growing on the agar, tubes were placed in the gradient. The condition and position of the amoebas were recorded daily. For the results to be accepted as valid, amoebas in two or three tubes had to start growth at 23° to 24°C and, in the gradient, grow at a lower temperature and fail to grow at a temperature 1° higher, with the same result when the experiment was repeated at a different time.

The strains of *Naegleria* tested (Fig. 1) formed two distinct groups. Amoebas fatal to humans grew well above the temperature of the highest fever; nonpathogenic amoebas did not grow above normal human temperatures.

Acanthamoeba strains showed a similar relationship; the strain most virulent to mice tolerated the highest temperature, while five nonpathogenic strains stopped growing at 37°C or below.

Hartmannella agricola (Fig. 1U) grows in tissue culture (with no cytopathic action) but is not pathogenic to mice (*13*). *Tetramitus rostratus* (Fig. 1V), which was tested by Culbertson (*13*), was nonpathogenic or, on prolonged testing, had a slight local effect. *Acanthamoeba rhysodes* (Fig. 1N) is more virulent to mice than *H. agricola* and *T. rostratus* despite its lower tolerance for high temperatures, perhaps because of the nutritional adaptability of *Acanthamoeba* species. The barrier to pathogenicity of *Hartmannella* and *Tetramitus* apparently is not related to temperature.

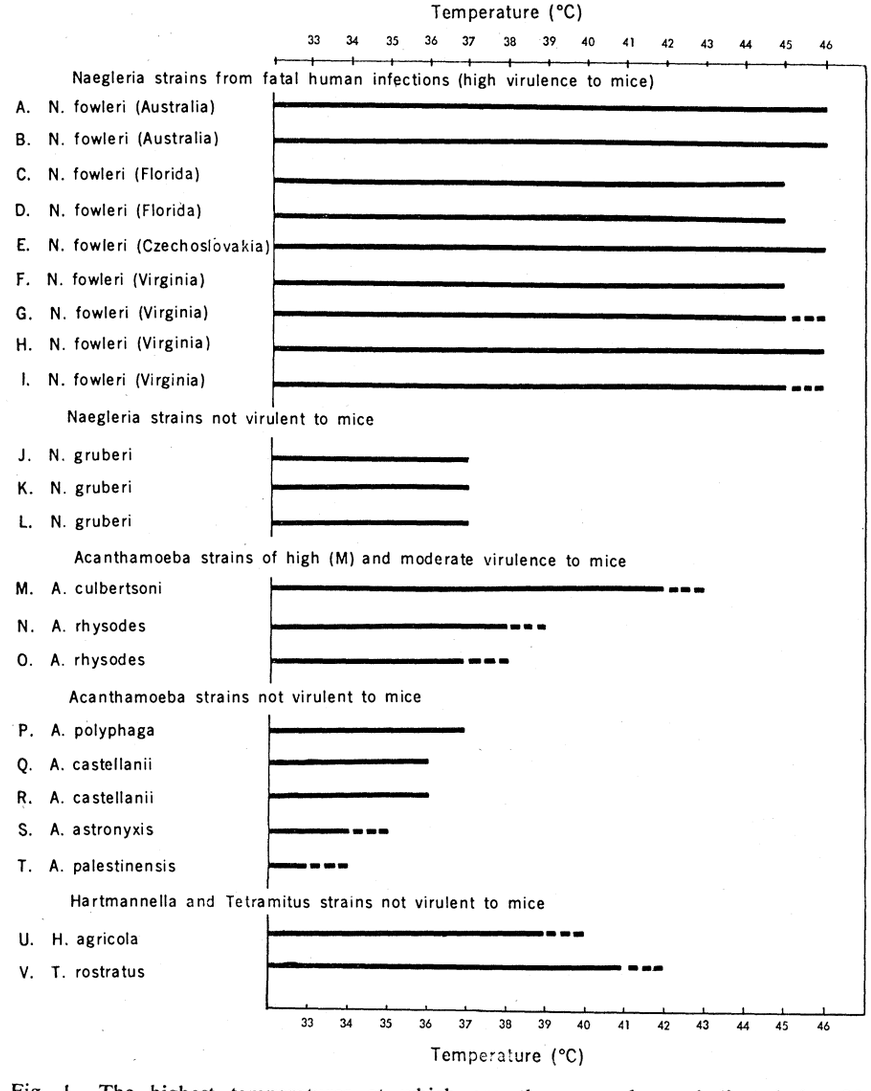

Fig. 1. The highest temperatures at which growth occurred are indicated for 22 strains of amoebas of known virulence or nonvirulence to humans or mice. Brief growth only on the first day was not counted as significant (*18*). The dotted lines indicate multiplication that seemed to be significant but did not reproducibly cover the slant in the usual way. The sources of the strains are given in (*19*), the strain numbers in (*20*), and published information on pathogenic effects in (*21*).

To be able to cause fatal primary amoebic meningoencephalitis, an amoeba must be able to multiply in the host and to survive fever, as minimal requirements. The relative importance of other factors (*3, 4*) is unknown.

Some parts of the body are cooler than the brain, and amoebas in granulomas (*3, 14*) or pneumonia (*15*) form cysts, which might survive episodes of fever and later excyst to cause recurrences. Thus, even an amoeba that grows only below 36°C could, in theory, cause disease.

In identifying *Naegleria* pathogenic for man, temperature tests may prove more useful than tests in mice. The strains of mice normally used were selected to be susceptible (*8. 9*) but differ in susceptibility. As one example, amoeba strains HN-3 (Fig. 1N) and A-5 (Fig. 1O) of Culbertson both killed the mice used by Singh and Das (*8*), whereas only HN-3 killed the mice used by Culbertson *et al.* (*3, 9*). A strain of *N. gruberi* that tolerates a temperature a little higher than do the strains tested (Fig. 1, J to L) might well kill a sensitive mouse but be no threat to humans. Singh and Das (*16*) isolated *Naegleria* strains only moderately pathogenic to mice; a temperature test might help resolve their identity and potential for causing human infections.

Many attempts have been made to isolate pathogenic *Naegleria* from swimming places linked to human infections, but few of the attempts have been successful. Nelson (*6*) isolated *N. fowleri* from a pond in Virginia in 1971. Anderson and Jamieson (*7*) in Australia obtained isolates from tap water and from a chlorinated pool, where there were probably few competing species of amoebas.

Because of the high optimal temperature of *N. fowleri*, incubation of environmental samples at room temperature favors rapidly growing nonpathogenic strains. For example, at 23°C, *N. gruberi* can grow over an agar slant in 1 day; *N. fowleri* can take from 1 week to 2 months. A procedure for selectively enhancing the growth of *N. fowleri* was devised and tested (*17*).

The results reported here add to the taxonomic diagnosis of *N. fowleri* (*5*): They are able to grow at 45°C with *Escherichia coli* on sparse nutrient agar; they transform readily to the flagellate form at 43°C; on sparse nutrient agar with *E. coli*, a population front advances at 1 to 3 mm/day at 23°C, 7 to 14 mm/day at 37°C, and 7 to 18 mm/day at 43°C (from four measurements at each temperature for each of nine strains).

Since *N. fowleri* was first obtained in culture with bacteria (*3, 4*), it has seemed that the amoebas might increase with bacterial pollution. The fact that human infections have almost all occurred during hot weather and after swimming in warm water (*3, 4*), also suggested a relationship to thermal pollution. My results suggest that combined coliform and thermal pollution would stimulate the growth of *N. fowleri*, but environmental data are lacking (*6, 7*).

Naegleria produced by pollution seemed to be a threat to individuals rather than to populations, because areas with increased coliforms are usually closed to water sports, but the reports by Anderson and Jamieson (*7*) give reason for concern. The tap water in Australia from which they isolated *N. fowleri* was chlorinated river water piped 300 km overland in the summer, and they could not, by superchlorination, remove *Naegleria* from a swimming pool in which human infection occurred. Environmental testing and sampling now seems more urgent and the procedures for differential culturing and quick identification (*17*) should be useful.

JOE L. GRIFFIN

Armed Forces Institute of Pathology, Washington, D.C. 20305

References and Notes

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2. Although experimental meningoencephalitis caused by *Acanthamoeba* was reported in 1958 [C. G. Culbertson, J. W. Smith, J. R. Minner, *Science* **127**, 1506 (1958)], the taxonomy remained confused until F. C. Page [*J. Protozool.* **14**, 499, 709 (1967)] clarified the distinctions between *Acanthamoeba* and *Hartmannella*. Singh and Das (*8*) placed *A. culbertsoni* in the genus *Hartmannella*, from which it must be transferred. The amoeboid trophozoites (the stage that invades) of these two genera differ. Since many kinds of amoebas and metazoan cells exhibit "hartmannella-like" typical spindles, groups based on this character are so all-inclusive as to be of little use.
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5. Of the three names applied to pathogenic *Naegleria*, *N. fowleri* Carter, 1970 [R. F. Carter, *J. Pathol.* **100**, 217 (1970)] was published first. The names *N. aerobia* Singh and Das, 1970 (*8*) and *N. invades* Chang, 1971 [S. L. Chang, in *Current Topics in Comparative Pathobiology*, T. C. Cheng, Ed. (Academic Press, New York, 1971), vol. 1, pp. 201–254] are nonvalid junior synonyms.
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12. Amoebas were grown on slants of Griffin sparse nutrient agar (GSNA) with *Escherichia coli* (strains A to O, Q, U, and V) or *Aerobacter* sp. (strains K, L, P to T and V). Strains K, L, Q, and V had the same temperature tolerance on either bacterium. Details of the culture method will be furnished on request (J. L. Griffin, in preparation).
13. C. G. Culbertson, personal communication.
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15. J. N. Dwivedi and C. M. Singh, *Indian J. Microbiol.* **5**, 31 (1965); E. E. McConnell, F. M. Garner, J. H. Kirk, *Pathol. Vet.* **5**, 1 (1968).
16. B. N. Singh and S. R. Das, *Curr. Sci.* **41**, 277 (1972).
17. Samples were incubated at 43°C to prevent the growth of other amoebas and allow growth of *N. fowleri*. All nine strains of *N. fowleri* formed numerous flagellates on dilution at 43° and 37°C. Information on methods for selective environmental isolation and differentiation will be furnished on request (J. L. Griffin, in preparation).
18. *Naegleria gruberi* grew so rapidly that tubes were put in the gradient immediately after subculture to a slant previously coated with bacteria and left overnight. In this way, 1-day growth could be distinguished from continuing growth.
19. The amoebas listed in Fig. 1 were obtained from the following sources: (strains A and B), R. F. Carter; (C, D, E, M, N, and U), C. G. Culbertson; (F, G, H, and I), E. C. Nelson; (J and O), R. G. Zieg, American Type Culture Collection, Rockville, Maryland; (K, L, Q, R, S, T, and V), Cambridge Culture Collection of Algae and Protozoa, Botany School, Cambridge, England; (P), T. K. Sawyer.
20. Following are the labels and sources of the labels for the strains in Fig. 1. The initials refer to names in (*19*). A, Nf-66 (R.F.C.); B, Nf-69 (R.F.C.); C, HB-1 (C.G.C.); D, HB-2 (C.G.C.); E, HB-3 (C.G.C.); F, MCV-TY-1 (Medical College of Virginia, E.C.N.); G, MCV-Lee-1 (E.C.N.); H, MCV-CJ-1 (E.C.N.); I, MCV-WM-1 (E.C.N.); J, L1-L (L1 from D. C. Warhurst, reisolate by S. L. Chang); K, 1518/1S (C.C.C.A.P.); L, 1518/1D (C.C.C.A.P.); M, A-1 (C.G.C.); N, HN-3 (C.G.C.); O, A-5 (C.G.C.); P, OX-1 (T.K.S.); Q, 1501/1 (C.C.C.A.P., Neff strain); R, 1501/2 (C.C.C.A.P.); S, 1534/1 (C.C.C.A.P.); T, 1547/1 (C.C.C.A.P.); U, 320G (probably S. S. Wang and H. A. Feldman); V, 1581/1 (C.C.C.A.P.).
21. Isolations showing virulence to humans of strains in Fig. 1: A (*3*, ref. 13); B (*3*, ref. 15); C (*3*, ref. 31), isolated from the same patient as D; D (*3*, ref. 10); E (*3*, ref. 18); F, I [R. J. Duma, W. I. Rosenbloom, R. F. McGehee, M. M. Jones, E. C. Nelson, *Ann. Intern. Med.* **74**, 923 (1971)]; G (*3*, ref. 38); H (*3*, ref. 12). References to tests in mice: J [D. C. Warhurst, *Trans. Roy. Soc. Trop. Med. Hyg.* **66**, 212 (1972)]; K and L (*8, 3*). In the latter, strain numbers are not given, but both studied *N. gruberi* from W. Balamuth presumably L) and others; K is listed by the C.C.C.A.P. as used by Singh. M (*2, 3, 9*); N (*8, 9*); O (*3, 8, 13*). Singh and Das found O moderately virulent, Culbertson found it nonvirulent. P (*3*, ref. 68); Q and R (*3*, ref. 29; 8); S and T, (*8*). Strain numbers are not given for S and T; apparently there is only one strain of each in the public domain. U and V (*13*).
22. Supported in part by research contract 3A062110A822 from the Army Medical Research and Development Command.

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