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Application of Flow Cytometry to Studies of Pathogenic Free-Living Amoebae†

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Species of small, free-living amoebae of the genera *Naegleria* and *Acanthamoeba* can cause fatal amoebic meningoencephalitis. Previous investigations have shown that pathogenic amoebae are associated with thermally altered water. Flow cytometric techniques for identifying species of pathogenic and nonpathogenic amoebae from such water have been developed, using immunofluorescence and fluorescein-bound concanavalin A. Flow cytometry is accomplished with a cytofluorograph, in which cells are dispersed in a suspended carrier liquid and passed in front of a focused argon ion laser beam. Cells are then distinguished by the degree of scattered light (size) or fluorescence. Flow cytometry techniques have proven efficient for environmental samples, as indicated by the identification of pathogenic *Naegleria fowleri* and nonpathogenic *Naegleri gruberi* and *Acanthamoeba castellanii* isolated from the Savannah River Plant in South Carolina. Cytofluorographic analysis of environmental samples has several advantages over the current methods of isolation and classification of free-living amoebae. With this system, it is possible to rapidly identify species and quantitate mixtures of pathogenic amoebae in environmental samples. Cytofluorographic analysis of amoebic isolates reduces the time presently required to screen environmental sites for pathogenic amoebae. The cytofluorograph permits detection and species identification of nonthermophilic *Naegleria* spp. and *Acanthamoeba* spp. that could not easily be isolated for species identification by conventional methods. Other advantages of flow cytometry over fluorescent microscopy include a high degree of statistical precision due to the large numbers measured, high immunofluorescent titers, and elimination of subjectivity and fluorescence fading.

Small, free-living amoebae are a group of aerobic protozoans found living independently in soil and aquatic environments. It has been discovered only within the last 2 decades that free-living amoebae can be the etiological agents of primary meningoencephalitis, chronic meningoencephalitis, pneumonitis, and conjuctivitis (1–3, 8, 10, 14, 16, 19, 26, 28; E. Willaert, Fed. Proc. 35:7, 1976). The clinical significance of these organisms has prompted an increased interest in determining the prevalence of pathogenic amoebae in their natural environment.

Griffin (13) reported that the optimal growing temperature for pathogenic *Naegleria fowleri* is 37°C; however, the protozoan can grow at temperatures as high as 45°C. Recent investigations (11, 13, 22, 23; R. L. Tyndall, E. L. Domingue, E. Willaert, and A. R. Stevens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, Q69, p. 231; R. L. Tyndall, C. B. Fliermans, and E. Willaert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N49, p. 187) indicate that natural or artificial heating of water may facilitate the propagation or persistence (or both) of *N. fowleri* and pathogenic *Acanthamoeba* spp. Swimming in thermally altered water has resulted in over 100 clinically reported cases of primary amoebic meningoencephalitis within the last 2 decades (30).

The public health problems presented by these organisms indicate the need for more studies to qualitate and quantitate pathogenic amoebae in heated environments. However, techniques for detecting, classifying, and quantitating these pathogens in environmental samples are laborious or nonexistent. The selective techniques for the isolation and identification of thermophilic pathogenic *Naegleria* spp. also se-

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lect for other thermophilic nonpathogenic *Naegleria* spp., and to date, there is no selective procedure to isolate pathogenic *Acanthamoebae* spp.

Techniques such as electrophoretic analysis of isoenzyme patterns (24), immunoelectrophoresis (31, 32; T. Kilpatrick and E. Willaert, J. Cell. Biol. **70**:113a, 1976), and indirect immunofluorescent microscopy (15, 20, 22, 32) have been used to classify species of free-living amoebae. Electrophoresis and immunoelectrophoresis are excellent techniques for classification of amoebae into subspecies; however, these techniques can be applied only to homogenous populations of amoebae and, therefore, would not be practical when analyzing environmental samples that contain more than one species of amoeba. Indirect immunofluorescent microscopy has been used effectively to classify free-living amoebae and, theoretically, can be used to identify a species of amoebae in a complex mixture with other species. However, since only a limited number of cells can be processed manually, fluorescent microscopy lacks statistical precision.

To aid in the classification of free-living amoebae taken from their natural habitat and to assist in the elucidation of the ecological parameters that select for the growth of the pathogenic species, we developed efficient and reliable flow cytometric methods for the identification of pathogenic and nonpathogenic amoebae.

In our technique, amoebae are measured by single-cell analysis (17) in a flow microcytometric system (Cytofluorograf model 4800A; Bio/-Physics Systems, Inc., Mahopac, N.Y.). Scatter and fluorescent signals are generated in the instrument as cells pass single file through a transparent flow channel and cross a 488-nm argon ion laser excitation beam. The fluorescein-conjugated immunoglobulins or lectins that are bound to the cells reemit fluorescent light in the green wavelength. This reemitted fluorescence is filtered to remove extraneous fluorescence, detected and amplified by photomultiplier tubes, and then displayed in the form of cytogram or histogram patterns. The cluster of dots seen in the cytogram patterns is representative of cell size (scatter) and degree of fluorescence. These data are stored in a multichannel distribution analyzer (model 2101; Bio/-Physics Systems), and the histogram distribution for the cells indicates fluorescent frequency. The cytofluorograph also has a built-in selected-count capacity in which a selected window can be formed on the cathode ray oscilloscope, which displays the cytogram patterns, thus allowing the percentage of a species of amoeba in the total population of cells to be determined.

MATERIALS AND METHODS

Amoeba Cultures. Stock cultures of amoebae were grown axenically. All *Acanthamoebae* spp., except *A. royreba*, were grown exclusively in a Casitone-based medium supplemented with 5 or 10% fetal calf serum, as described previously (29). *A. royreba* was cultured in Casitone-based medium or Plus I medium (Industrial Biological Laboratories, Rockville, Md.) supplemented with 10% fetal calf serum. *Naegleria* spp. were grown in a similar Casitone-based medium with 10% fetal calf serum but without sodium chloride. Stock cultures of *N. fowleri, N. lovaniensis, A. royreba, A. culbertsoni*, and *Acanthamoeba* sp. isolate 5334 (isolated from heated water of the Savannah River Plant, Aiken, S.C., by R. L. Tyndall and E. L. Domingue [unpublished data]) were maintained at 37°C. All other cultures were grown at 28°C.

Amoebae cultured for growth experiments or used directly from environmental samples were grown on live *Escherichia coli* by two procedures. In the first procedure, 0.6-ml portions of an *E. coli* suspension (10^9 bacteria per ml) were spread evenly over the total surfaces of 2% nonnutrient agar plates (100 by 15 mm), which were stored at 4°C for no more than 1 week before use. The amoebae or the environmental samples were placed in the center of *E. coli* plates and incubated at 28, 37, or 45°C, depending upon the species or the experimental requirements. The second procedure consisted of mixing the *E. coli* and amoebae, spreading this mixture in an even lawn over the total surface of the nonnutrient agar plates, and incubating the plate immediately.

Production of antisera. Antisera were prepared by intravenously injecting portions of live amoebae (10^6) into the lateral ear veins of New Zealand white rabbits, as previously described by Willaert (Fed. Proc. **35**:7, 1976). Antisera were prepared against the following species of free-living amoebae: *N. fowleri, N. lovaniensis, N. gruberi, A. culbertsoni, A. royreba, A. castellanii* (Neff), *A. rhysodes* (CCAP), *Acanthamoeba* sp. isolate 5334, and *Acanthamoeba* sp. isolate A3 (strains 5334 and A3 were isolated by R. L. Tyndall and E. L. Domingue [unpublished data]).

Flow cytometric indirect immunofluorescent staining and analysis. Exponentially growing 24-h cells were harvested and concentrated by centrifugation. The suspended amoeba pellet was fixed with 0.2 M sucrose–10% formaldehyde in a phosphate buffer for 30 min at 4°C. Centrifuging and removing the fixative enabled incubation of the amoebae in diluted antiserum for 20 min in a 37°C water bath. The antiserum was then removed by centrifugation, and the cells were washed twice with isotonic phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.2). Finally, the cells were incubated with diluted fluorescein-bound goat anti-rabbit immunoglobulins (Cappel Laboratories, Inc., Cochranville, Pa.) for 20 min at 37°C in the dark and were then washed.

Amoebae were kept at 1×10^5 to 5×10^5 cells per ml, and the assay volume was 1 ml. Cells were concentrated by centrifugation at $150 \times g$ for 3 min. Isotonic PBS was used as the diluent and wash.

To determine the percentage of fluorescing amoebae from the percentage of nonfluorescing amoebae, the selected-count controls, scatter gains, and fluorescent

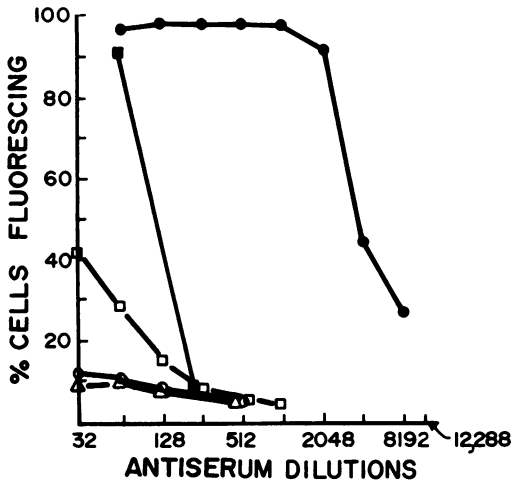

FIG. 1. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *A. culbertsoni* (A1). Anti-*A. culbertsoni* antiserum (●), anti-*A. castellanii* (Neff) antiserum (△), anti-*A. royreba* antiserum (□), anti-*A. rhysodes* antiserum (■), and preimmune serum (○) were used.

gains were held constant for all immunofluorescent readings. For each sample, the means and standard deviations of five percentiles were calculated. Each percent was generated from 1,000 cells.

Cell binding and analysis of fluorescein-labeled concanavalin A. Amoebae fixed with sucrose-formaldehyde were tagged with fluorescein-labeled concanavalin A (ConA-F; Calbiochem-Behring Corp., La Jolla, Calif.) by incubating the cells with ConA-F at room temperature for 20 min. The binding reaction consisted of 0.025 mg of ConA-F per ml in 1 ml of isotonic PBS containing 1×10^5 to 5×10^5 cells. After binding, the cells were washed once with 5 ml of PBS and assayed flow cytometrically for fluorescence.

All instrumentation settings were held constant for ConA-F analysis as well as for immunofluorescent analysis; however, the green gain was readjusted for each set of control samples.

Environmental sampling. Environmental samples were taken from Savannah River Plant sites that have previously been shown to contain pathogenic *N. fowleri* (12; R. L. Tyndall, C. B. Fliermans, and E. Willaert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N49, p. 187). Thirty-five samples were taken in January 1980 from four sites.

Aquatic plants, mud, and detritus were gathered from the littoral zone of the ponds and the bottom of artificial stream 6 (a greenhouse facility) and were immediately placed in separate sterile containers. Within 2 h of collection, approximately 1 ml of each sample was placed in the center of each nonnutrient agar plate seeded with *E. coli*. The plates were sealed with parafilm, and all but five were incubated at 45°C for the selective growth of thermophilic *Naegleria* spp. Five plates from artificial stream 6 were incubated at 28°C for the isolation of nonthermophilic *Naegleria* spp. and *Acanthamoeba* spp. Daily observations of the plates for the presence and morphology of outgrowths were made with an inverted microscope. The amoebae that grew and migrated away from the debris at the point of application of the environmental samples were harvested with sterile 0.25% PBS (NaCl). A low salt concentration (e.g., 0.25% NaCl) increases the viability of *Naegleria* spp. (20). The amoebae were then mixed with *E. coli*, and the mixtures were spread evenly over the entire surfaces of nonnutrient agar plates. After 24 h of growth, the trophozoites were harvested for further analysis.

Amoebae that grew at 45°C were examined flow cytometrically with anti-*N. fowleri* antiserum and ConA-F for the presence of *N. fowleri* and *N. lovaniensis*. Outgrowth at 28°C was analyzed by flow cytometry with a battery of five antisera for preliminary species identification of nonthermophilic amoebae. Preimmune and homologous antisera prepared against known amoebae were run simultaneously with each environmental sample to calculate the theoretical percentage of a species of amoebae. The percent background fluorescence seen with the preimmune sera was subtracted from the percent fluorescence seen with all samples and homologous controls (background fluorescence was usually <5%). With each experimental run, a ratio was obtained by dividing 100% by the observed percentage of amoebae that fluoresced with the homologous antisera (observed percentage for controls was usually greater than 95). This ratio was multiplied by the percentage of cells that fluoresced in an environmental sample to calculate a theoretical percentage of a species in that sample, i.e., 100/[percent homologous fluorescence − background fluorescence) × (percent fluorescence of environmental sample − background fluorescence)].

RESULTS

Flow cytometry, agglutination, and microscopic immunofluorescent titers. In the development of the indirect immunofluorescent flow microcytometry procedure, it was determined that the best overall results were obtained by fixing cells with sucrose-formaldehyde before immunological treatment and by using isotonic PBS as the wash solution and diluent for the antisera and for the fluorescein-bound immunoglobulin. These assay conditions gave a moderate cell recovery (71.8 ± 2.9% mean ± standard error for five free-living amoebal species), concise cytogram patterns, good separation of frequency distribution histogram peaks from the origin, and a relatively low background fluorescence. Incubation with antiserum at 37°C for over 20 min caused an increase in nonspecific fluorescence. Figures 1 to 7 illustrate the homologous and heterologous antisera titrations obtained cytofluorographically when antisera were reacted against various amoebae.

N. lovaniensis is antigenically similar to *N. fowleri*, differing in antigenic specificity by only one dilution (Fig. 5 and 6). When anti-*N. fowleri* antiserum was twice absorbed with methanol-fixed *N. lovaniensis*, a maximal separation of clusters representative of homologous and het-

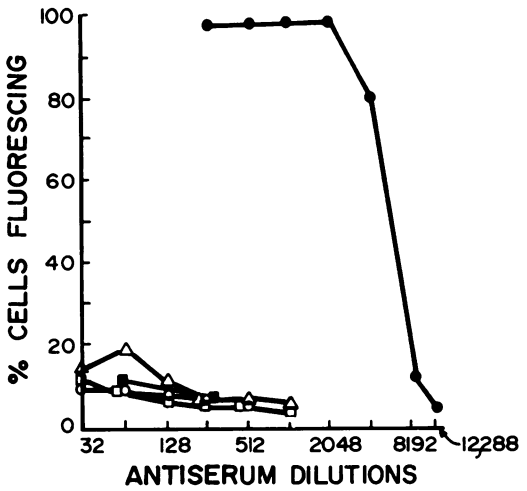

FIG. 2. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *A. royreba*. Anti-*A. royreba* antiserum (●), anti-*A. castellanii* antiserum (△), anti-*A. culbertsoni* antiserum (□), anti-*A. rhysodes* antiserum (■), and preimmune serum (○) were used.

erologous fluorescence occurred at a dilution of 1:64 (Fig. 8). The separation of these normally fused cytogram patterns enabled an easy differentiation between *N. fowleri* and *N. lovaniensis* to be made. Absorbing anti-*N. fowleri* antiserum with *N. fowleri* abolished the reactivity of the antiserum to *N. fowleri* and *N. lovaniensis*.

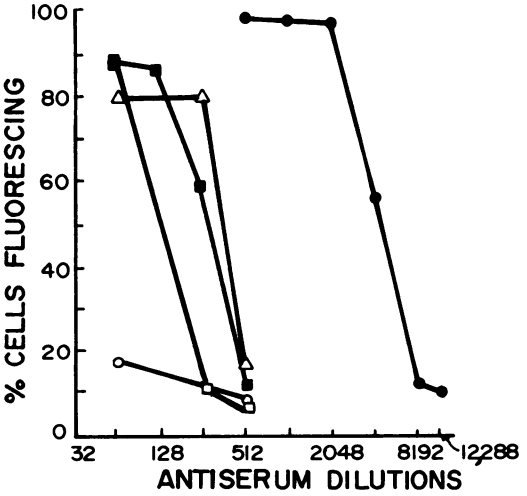

FIG. 3. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *A. castellanii* (Neff). Anti-*A. castellanii* antiserum (●), anti-*A. culbertsoni* (A1) antiserum (△), anti-*A. royreba* antiserum (□), anti-*A. rhysodes* antiserum (■), and preimmune serum (○) were used.

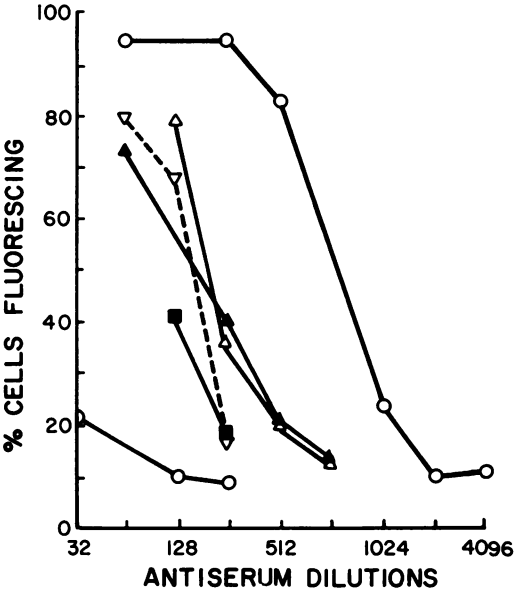

FIG. 4. Titrations of homologous and heterologous antisera with the antigen *A. rhysodes* (CCAP). Anti-*A. rhysodes* (CCAP) antiserum (●), anti-*A. royreba* antiserum (■), anti-*A. castellanii* (Neff) antiserum (△), and control serum (○) were used. Anti-*A. rhysodes* antiserum was titrated with *A. polyphaga* (APG) (▽).

Agglutination and indirect immunofluorescent microscopy were also used to determine the homologous and heterologous titers of antisera prepared against selected species of amoeba (27). These two techniques were found to be less

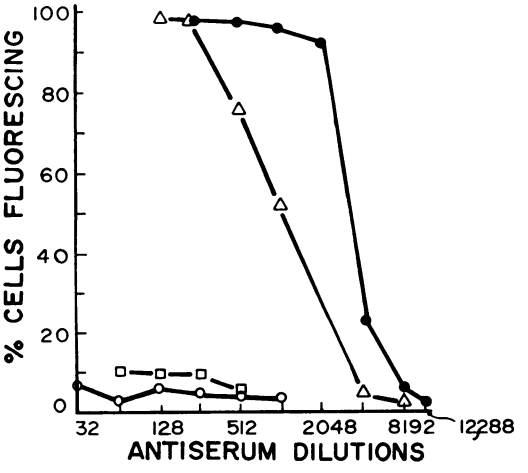

FIG. 5. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *N. fowleri*. Anti-*N. fowleri* antiserum (●), anti-*N. lovaniensis* antiserum (△), anti-*N. gruberi* antiserum (□), and preimmune serum (○) were used.

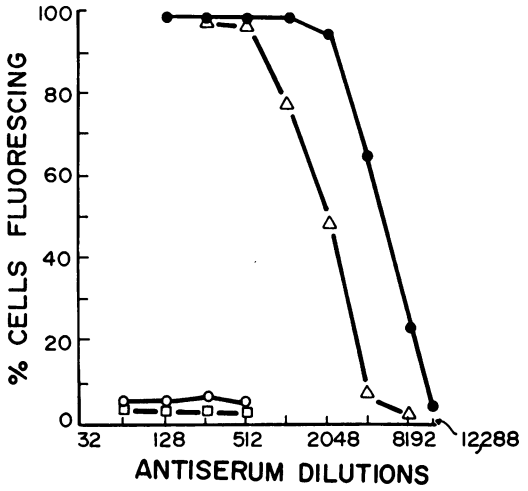

FIG. 6. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *N. lovaniensis*. Anti-*N. lovaniensis* antiserum (●), anti-*N. fowleri* antiserum (△), anti-*N. gruberi* antiserum (□), and preimmune serum (○) were used.

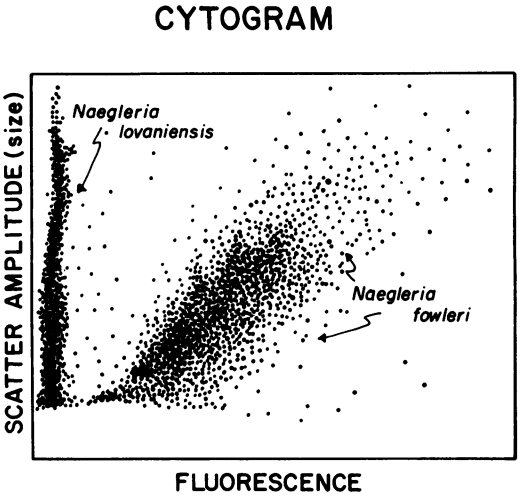

FIG. 8. Cytogram pattern seen with monospecific anti-*N. fowleri* antiserum when *N. fowleri* and *N. lovaniensis* were mixed in the same culture. Anti-*N. fowleri* antiserum was absorbed twice with a cell-to-serum volume ratio of 1:2 (for 45 min at 37°C) with (cold) methanol-fixed *N. lovaniensis*. The antiserum dilution was 1:64.

sensitive than flow cytometry in the determination of antiserum titers (Table 1). The endpoint agglutination titers for all homologous reactions ranged from 1:128 to 1:256, whereas heterologous reactions were equal to or less than a 1:2 dilution. Endpoint titers of homologous microscopic immunofluorescent assays were generally higher than agglutination titers, ranging from 1:256 to 1:2,048. Heterologous microscopic fluorescence for all species was at least one dilution less than homologous fluorescence. Flow microfluorometric titers were, on the average, 10- to 20-fold higher than the agglutination titers and 2- to 10-fold higher than the microscopic immunofluorescent titers.

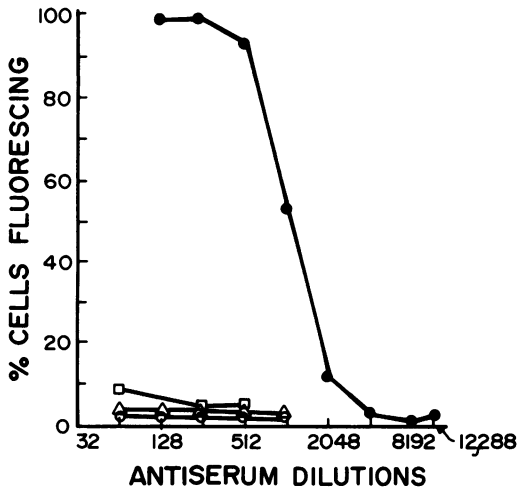

FIG. 7. Flow microfluorometric titrations of homologous and heterologous sera with the antigen *N. gruberi*. Anti-*N. gruberi* antiserum (●), anti-*N. fowleri* antiserum (△), anti-*N. lovaniensis* antiserum (□), and preimmune serum (○) were used.

ConA-F. By utilizing ConA-F and by assaying flow cytometrically, we derived a useful test for distinguishing between the antigenically similar *N. fowleri* and *N. lovaniensis*. Titration of *N. fowleri* and *N. lovaniensis* with ConA-F gave the greatest separation between clusters representing fluorescing and nonfluorescing cells at a lectin concentration of 0.025 mg/ml. The fluorescent cluster of *N. lovaniensis* was adequately separated from the nonfluorescent cluster of *N. fowleri*, which allowed for an accurate cytofluorographic determination of the percentage of *N. lovaniensis* in a mixture with *N. fowleri* (Table 2).

Competitive and relative growth experiments. Normally, environmental samples contain nonpathogenic thermophilic amoebae growing in competition with pathogenic amoebae. To determine if the flow cytometric procedures could accurately differentiate subpopulations of amoebae, we mixed two species of amoeba in known concentrations before immunological reactions. The percentage of fluorescing amoebae correlated well with the percentage in the test mixture (Table 3).

Observations by De Jonckheere and Van de

TABLE 1. Comparison of flow cytometry fluorescence with fluorescent microscopy and agglutination

Species and antiserum	Technique used	Amt of fluorescence or agglutination with antiserum titration of[a]:									
		1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	1:12,288
N. fowleri + anti-*N. fowleri*	Flow cytometric fluorescence[b]			+4	+4	+4	+4	+4	+2	+1	0
	Fluorescent microscopy[c]	+4	+3	+3	+2	+2	+2	+1	0	0	
	Agglutination[d]	+	+	+	0	0	0				
A. castellanii + anti-*A. castellanii*	Flow cytometric fluorescence				+4	+4	+4	+4	+2	+1	0
	Fluorescent microscopy	+3	+3	+3	+3	+2	+2	0	0		
	Agglutination	+	+	+	+	0	0				
A. culbertsoni + anti-*A. culbertsoni*	Flow cytometric fluorescence		+4	+4	+4	+4	+4	+4	+2	+2	0
	Fluorescent microscopy	+4	+3	+2	+1	0	0				
	Agglutination	+	+	+	0	0	0				
A. royreba + anti-*A. royreba*	Flow cytometric fluorescence				+4	+4	+4	+4	+4	+1	0
	Fluorescent microscopy	+4	+3	+3	+2	0	0				
	Agglutination	+	+	+	+	0	0				

[a] For flow cytometry fluorescence, the +4 to 0 scale represents the percentage of cells that fluoresced with the selected-count controls held constant: +4, 75 to 100% of the amoebae gave fluorescent signals that fell within the selected window; +3, 50 to 75%; +2, 25 to 50%; +1, background to 25%; 0, equal to or below background. For fluorescent microscopy: +4, highest degree of fluorescence; +3, good degree of fluorescence; +2, moderate degree of fluorescence; +1, low degree of fluorescence; 0, no fluorescence. For agglutination: +, agglutination present; 0, no agglutination present.

[b] As the antiserum concentration was decreased, the cytogram clusters moved to the origin of the fluorescent axis, thereby indicating a relative loss in degree of fluorescence.

[c] The diluted antisera were incubated (15 min at 37°C) on slides containing air-fixed amoebae, which were then washed and exposed to a 1:100 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin. The slides were again washed in PBS and examined for degree of fluorescence.

[d] Exponentially growing amoebae were harvested, washed, and added to serial dilutions of antiserum in flat-bottom microtiter tissue culture plates.

Voorde (9) of the growth pattern of *Naegleria* strains on *E. coli*-seeded nonnutrient agar plates indicate that thermophilic *N. lovaniensis* has a relative growth advantage over *N. fowleri*. Competition experiments reported herein quantitated the apparent growth advantage of *N. lovaniensis* over growth of thermophilic *N. fowleri* and *Acanthamoeba* spp. at 37°C and 45°C.

TABLE 2. *Naegleria* and *Acanthamoeba* fluorescence with ConA-F at 0.025 mg/ml

Amoeba	% of fluorescent cells[a]
A. castellanii (Neff)	3.7 + 0.3
A. culbertsoni (Al)	5.6 + 0.2
A. royreba	5.0 + 0.4
A. polyphaga (APG)	4.9 + 0.2
Acanthamoeba sp. isolate 5334	13.3 + 0.2
N. fowleri	5.7 + 0.3
N. gruberi	8.5 + 0.3
N. lovaniensis	91.6 + 0.7
50% *N. fowleri* + 50% *N. lovaniensis*	50.3 + 0.7

[a] Mean and standard error for one experiment.

Amoebae from environmental samples required 3 to 4 days to grow to the edge of the *E. coli*-seeded plates after initial planting. It was established that 10^5 amoebae seeded on a plate would grow to the edge in approximately 3 days; therefore, for all competition experiments, this number of amoebae was used in an attempt to simulate the growth rates of environmental amoeba isolates. The first series of competition experiments involved planting a known mixture of 10^5 amoebae in the center of replicate *E. coli* plates, harvesting daily, and assaying flow cytometrically for a change in percentage of each species. At 37°C, the net growth of *N. lovaniensis* was faster than that of *N. fowleri* (Fig. 9A). When *N. fowleri* and *N. lovaniensis* were mixed and incubated at 45°C, the number of active *N. lovaniensis* trophozoites exceeded that of *N. fowleri* within 48 h, 24 h less than the time required at 37°C (Fig. 9B). Also, the increase in percentage of *N. lovaniensis* within the first 24 h of culturing at 45°C was not seen at 37°C. Thus, increasing the incubation temperature from 37°C

TABLE 3. Determination of the percentages of amoebae in a mixed culture

Amoebic mixture	Reacted with:	% of fluorescing cells for the following ratio (%) of mixed cells[a]:						
		100:0	90:10	75:25	50:50	25:75	10:90	0:100
N. fowleri + *N. lovaniensis*	Anti-*N. fowleri* antiserum (diluted 1:64)[b]	100	94.8	80.5	54.4	33.5	14.7	0
N. gruberi + *N. lovaniensis*	Anti-*N. gruberi* antiserum (diluted 1:152)	100	89.7	77.8	54.9	28.9	14.5	0
N. fowleri + *N. lovaniensis*	ConA-F (0.025 mg/ml)	100	93.3	75.5	55.5	28.0	9.4	0
N. lovaniensis + *Acanthamoeba* sp. isolate 5334	ConA-F (0.025 mg/ml)	100	87.3	74.8	51.0	25.5	12.4	0

[a] Each value in this section represents the percentage of fluorescing cells calculated from the cytofluorograph reading and the correction formula as described in the text. The mean of each column is within the 90% confidence interval, as calculated by Student's *t* test.

[b] Anti-*N. fowleri* antiserum was absorbed with *N. lovaniensis*.

to 45°C facilitated the growth advantage of *N. lovaniensis*. Pathogenic *Acanthamoeba* sp. isolate 5334 was overgrown by *N. fowleri* and *N. lovaniensis* within the first 48 h when all three species were mixed and incubated at 37°C (Fig. 9C).

To analyze the noncompetitive growth rate of amoebae, we placed mixtures of 2.4×10^4 amoebae directly in *E. coli* suspensions. This *E. coli*-amoeba suspension was then spread evenly over the total surface of nonnutrient agar plates, which were incubated for 24 h. Within the first 24 h, a five- to sixfold increase in cell numbers occurred without greatly altering the percentages of mixed *N. fowleri* and *N. lovaniensis* (Fig. 10).

By spreading individual amoeba suspensions over the total surfaces of *E. coli*-seeded agar plates and incubating the plates at 45°C, the generation time of *N. fowleri* was shown to be 8.9 ± 0.1 h, and that of *N. lovaniensis* was shown to be 9.0 ± 0.5 h. Pathogenic *Acanthamoeba* sp. isolate 5334 did not grow at 45°C. At 37°C, the generation time of *N. fowleri* was 8.6 ± 0.2 h, that of *N. lovaniensis* was 12.9 ± 0.4 h, and that of isolate 5334 was 9.9 ± 0.4 h. By taking time points within the first 4 h and at 24 h after planting, it was determined that the lag phase for all three species at both temperatures was less than 1 h. Calculation of generation time was done by the method of Mitcheson (18).

Analysis of environmental samples. Flow cytometric species identification of free-living amoebae was tested on environmental samples taken at the Savannah River Plant. Four of five samples that gave positive cytogram patterns for *N. N. fowleri* but exhibited heterologous fluorescence, flagellation, or a morphological growth pattern which resembled that of *N. fowleri* were tested for pathogenicity in mice (Table 4). All four samples that gave a positive cytogram pattern were pathogenic for mice. Amoebae were reisolated from brain tissue and identified by flow cytometry as *N. fowleri*. The three samples that gave negative cytogram patterns for *N. fowleri* were not infectious for mice upon intranasal inoculation. *N. fowleri* was not detected in the artificial flowing stream 6 sample no. 4 when incubated at 28°C but was detected when incubated at 45°C.

Flow cytometric analysis of Hot Dam sample no. 4 suggested that 56% of the cells were reacting with the anti-*N. fowleri* antiserum (absorbed with *N. lovaniensis*). The cytogram pattern (Fig. 11A) indicated that this was due to heterologous fluorescence of an organism whose species had not been identified. Coordinates of the cytogram pattern from Hot Dam sample no. 5 were identical to those of the *N. fowleri* control (Fig. 11B).

The cytogram pattern of artificial stream 6 sample no. 4 (which gave only 0.23% fluorescence) was resolved by increasing the number of cells analyzed in the cytofluorograph. Generally, for each sample, the means and standard deviations of five percentiles were calculated. Each percent was generated from 1,000 cells. However, analysis of 15,000 cells consecutively gave a cytogram pattern for artificial stream 6 sample no. 4 identical to that of a known stock culture of *N. fowleri*.

Of the four samples that were incubated at 28°C, we flow cytometrically detected *A. castellanii* in two and *N. gruberi* in one (Table 5).

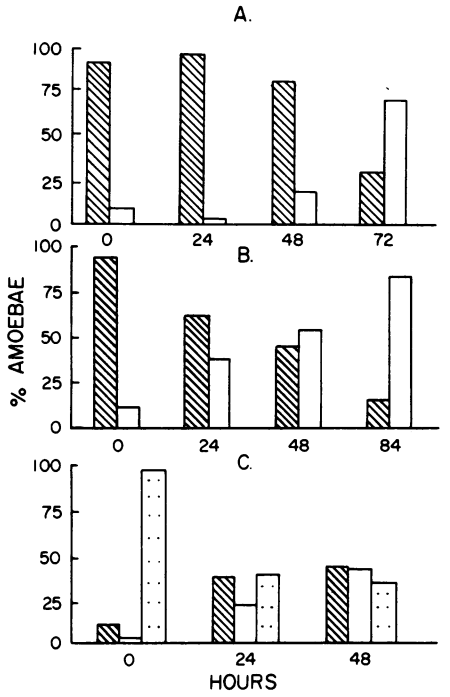

FIG. 9. Percent growth of *N. fowleri*, *N. lovaniensis*, and *Acanthamoeba* sp. isolate 5334 when the species were mixed and then planted in the centers of *E. coli*-seeded agar plates. The percentage of amoebae was determined with anti-*N. fowleri* antiserum (absorbed twice with *N. lovaniensis*) at a dilution of 1:64, the percentage of *N. lovaniensis* was determined with ConA-F, and the percentage of *Acanthamoeba* sp. isolate 5334 was determined with 5334 antiserum at a dilution of 1:256. (A and C) Incubation at 37°C; (B) incubation at 45°C. Symbols: ⊡, isolate 5334; ▩, *N. fowleri*; □, *N. lovaniensis*.

DISCUSSION

In recent years, environmental sampling of thermally altered waters for pathogenic amoebae has increased. Current methods for isolation and identification of pathogenic amoebae from the environment are quite laborious. At one sample site, many samples may be taken, and each plate must be monitored for morphological growth patterns indicative of pathogenic amoebae. Subsequent tests for flagellation and pathogenicity are then necessary. Even after isolation of the pathogens, either directly or via passage through infected animals, species identification is desirable. Methods most frequently used for final classification of amoeba isolates are morphological characterization of trophozoites and cysts, histochemical analysis, and indirect immunofluorescent microscopy (4, 5, 7, 10, 22, 24, 26, 31, 32; G. Saygi, Trans. R. Soc. Trop. Med. Hyg. **63**:11, 1969; E. Willaert, Fed. Proc. **35**:7, 1976).

Morphological species identification of free-living amoebae can be subjective and, when used alone, may be considered by some investigators to be inconclusive. Thus, fluorescent microscopic species identification must be done on brain isolates that have been axenized and cloned, which can be time consuming when identifying species in a large number of environmental samples. Also, with fluorescent microscopy, a high degree of cross-reactivity caused by unidentified, antigenically similar amoebae could be incorrectly interpreted as being due to the pathogen. In addition, prolonged exposure of the fluorochrome to the excitation wavelength in fluorescent microscopy results in fading of the sample. In contrast, the statistically sound methodology provided by flow cytometry for rapid species identification of pathogenic free-living amoebae from environmental samples permits large-scale screening which can be undertaken with ease and accuracy. The results reported in this study on the preliminary species identification of two nonpathogenic amoebae from the Savannah River Plant (*N. gruberi* and *A. castel-*

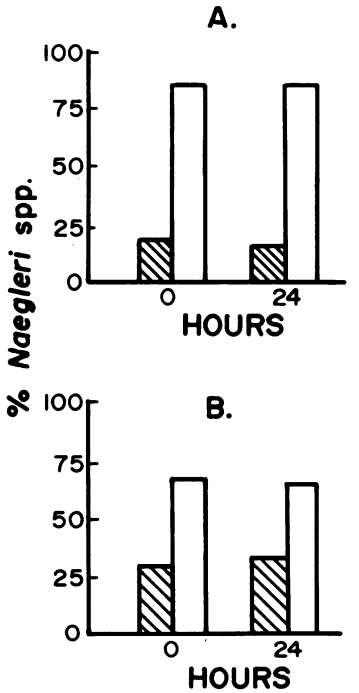

FIG. 10. Percent growth of *N. fowleri* and *N. lovaniensis* at 45°C when *E. coli* was mixed with the two species and the *E. coli*-amoeba mixture was spread evenly over the total surfaces of nonnutrient agar plates. The percentage of *N. fowleri* was determined with *N. lovaniensis*-absorbed anti-*N. fowleri* antiserum, and the percentage of *N. lovaniensis* was determined with ConA-F. Symbols: ▩, *N. fowleri*; □, *N. lovaniensis*.

TABLE 4. Mouse pathogenicity and flow cytometric analysis of environmental samples[a]

Sample site (isolate no.)	% Fluorescence[a]	Cytogram indicative of pathogenic *N. fowleri*	Flagellation[b]	Mouse pathogenicity[c]	% of *N. fowleri* from mouse brain
Hot Dam (2)	1.71	No	Yes	0	
Hot Dam (4)	56.04	No	No	0	
Hot Dam (5)	99.72	Yes	Yes	4	98.56
Hot Dam (1)	40.51	Yes	Yes	3	99.42
Byers Bay (5)	98.73	Yes	Yes	4	98.38
Flow Stream 6 (4)	0.23	Yes	Yes	4	98.20
Flow Stream 6 (4)[d]	67.66	No	No	0	

[a] The cytogram window was set to detect heterologous and homologous fluorescence with anti-*N. fowleri* antiserum.

[b] Flagellation tests were performed by suspending amoebae in sterile distilled water and incubating at 45°C for 1 to 3 h (25).

[c] For each sample, 10^4 amoebae suspended in sterile water were administered by intranasal innoculation into four anesthetized mice.

[d] Incubated at 28°C; all other samples were incubated at 45°C.

lanii) also strongly indicate the value of flow cytometry for immunotaxonomy. More importantly, flow cytometry may be used in the future for the identification of pathogenic *Acanthamoeba* spp. in environmental samples.

We demonstrated that flow cytometry could detect *N. fowleri* before pathogenicity tests. Use of flow cytometry could thereby decrease the number of false-negatives by decreasing the probability of some organisms not being detected. Conversely, the number of pathogenicity tests needed could also be reduced when a number of samples are shown by flow cytometric analysis to be devoid of *N. fowleri*. Also, statistical precision obtained by flow cytometry relative to the large sample size reduces subjectivity. With flow cytometry, homologously fluorescing cells can be differentiated from heterologously fluorescing cells by cytogram patterns. Also, with flow cytometry, the millisecond exposure of cells to the argon ion laser excitation beam prevents fading (17). Finally, the cytofluorograph is a very sensitive instrument for detecting fluorescence. A survey of the literature revealed that antibody titers detected by flow cytometry represented some of the highest fluorescence yet obtained with small, free-living amoebae (4, 20, 21, 22, 26, 31, 32).

Environmental samples often contain thermophilic, nonpathogenic *N. lovaniensis* growing in competition with pathogenic *N. fowleri*; consequently, the use of an *N. lovaniensis*-absorbed, anti-*N. fowleri* antiserum was required when looking for the pathogen. The high specificity of anti-*N. fowleri* antiserum permitted a sharp demarcation of cytogram patterns with the 1:64-diluted, absorbed antiserum. The use of antiserum at high concentrations (low dilutions) increased the possibility of cross-reactivity from other thermophilic organisms. Thermophilic, nonpathogenic *Naegleria* spp. and *Acanthamoeba* spp. (12, 22, 23; R. L. Tyndall, E. L. Domingue, E. Willaert, and A. R. Stevens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, Q69, p. 231; R. L. Tyndall, C. B. Fliermans, and E. Willaert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N49, p. 187) have been isolated from heated waters of >40°C. However, heterologous fluorescence from an unidentified organism could only be superimposed on the *N. fowleri* cytogram cluster if the cell surface area (proportional to scatter)-to-fluorescent ratios were identical. The above is unlikely and was not encountered in this study.

Equally valuable in distinguishing between the pathogenic and nonpathogenic thermophilic *Naegleria* spp. was the use of ConA-F. Low concentrations of ConA agglutinate *N. lovaniensis* but have no effect on *N. fowleri*. It has been stated that the degree of agglutination of mammalian cells with ConA does not necessarily correlate with the degree of ConA-F binding (6). The data reported here are the first to demonstrate that ConA-F bound to *N. lovaniensis* at a concentration at which it did not appreciably bind to *N. fowleri*, *N. gruberi*, or five species of *Acanthamoeba*. This quick and easy fluorescent assay with ConA-F may prove very useful in future studies of the environmental distribution of and competition between *N. fowleri* and *N. lovaniensis*.

Attempts at calculating the generation time as amoebae grow from the centers of *E. coli* plates to the outer edges would be difficult. As the active trophozoites move toward the peripheries of plates, a trail of cysts is left behind. Chang (5) reported that when trophozoites of *N. gruberi* begin to encyst, a period of slower growth,

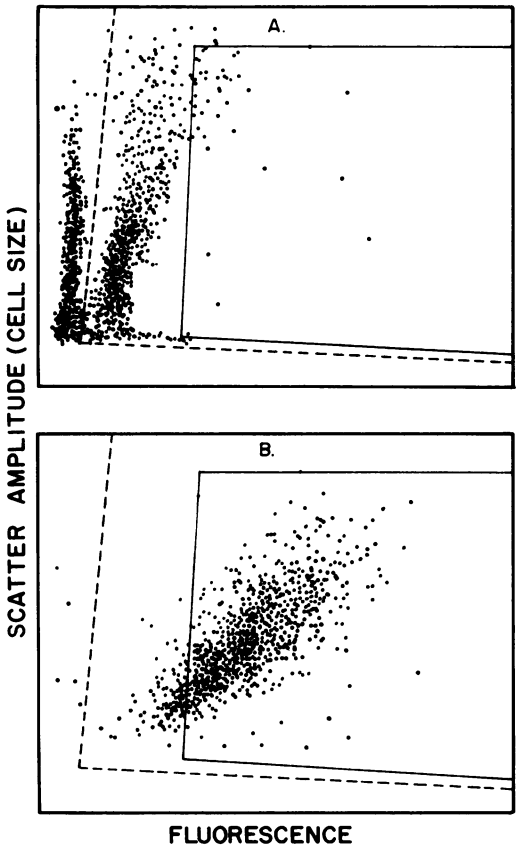

FIG. 11. Cytogram patterns of environmental samples no. 4 and no. 5 from Hot Dam in the Savannah River Plant. The solid lines designate the selected window (cytogram thresholds) set to detect the percentage of amoebae in the sample that exhibited homologous fluorescence. The dashed lines designate the window set to detect the percentage of fluorescing cells, whether homologous or heterologous. Dots to the left of the dashed line represent nonfluorescing amoebae. (A) Hot Dam sample no. 4 with 56% of the amoebae fluorescing, as indicated by the cluster of dots falling within the dashed lines. The window set for homologous fluorescence contains 6.3% of the dots representing fluorescing amoebae; however, this overlap was due to the wide distribution of heterologously fluorescing cells and not to the presence of *N. fowleri*. (B) Single cluster seen with Hot Dam sample no. 5 suggested the presence of a pure culture of *N. fowleri*.

related to the earlier logarithmic growth, is observed. In this period of slow growth, there are still logarithmically growing cells in the growth front. Calculations of relative growth rates of trophozoites in the front would also be difficult because active amoebae are often mixed with cysts and because different species encyst at different rates. Accurate generation times were calculated by dispersing amoebae evenly over the entire *E. coli* plate (to prevent intraspecies competition) and harvesting before the onset of encystment.

As reported here, *N. fowleri* exhibited the same generation time as *N. lovaniensis* at 45°C and a shorter generation time at 37°C; however, *N. lovaniensis* overgrew *N. fowleri* at both temperatures when both species were simultaneously seeded in the centers of *E. coli* plates. One explanation for the ability of *N. lovaniensis* to overgrow *N. fowleri* may be the low degree of intraspecies nutrient competition occurring with *N. lovaniensis*, since this species grows with a diffuse front rather than with a highly condensed front, such as that found for *N. fowleri*. Another possible explanation of why *N. lovaniensis* eventually overgrew *N. fowleri* at 37°C and 45°C is that dissemination of *N. lovaniensis* over the plate was faster than that of *N. fowleri*, thereby decreasing the number of available *E. coli* cells for the slow-moving *N. fowleri* front.

Even though the generation time of *N. fowleri* was less than that of *N. lovaniensis* at 37°C, this was not sufficient to imply that isolation of the pathogen should be made at 37°C instead of 45°C. At 37°C, large numbers of other organisms from the environmental samples began to grow, which further complicated the isolation of *N. fowleri*. The fact that *N. fowleri* was isolated from a sample incubated at 45°C and was not isolated from the same sample incubated at 28°C was suggestive of this point (Table 4).

As with *N. fowleri*, *Acanthamoeba* sp. isolate 5334 also had a shorter generation time than *N. lovaniensis*; however, when all three amoebae were mixed, the two *Naegleria* spp. overgrew isolate 5334 at 37°C. A possible explanation for this is that by the first 24 h, isolate 5334 began to encyst, thereby limiting its multiplication. The rate of encystment for *N. lovaniensis* was observed to be slower than that for isolate 5334. This was observed with pure cultures of both *N. lovaniensis* and isolate 5334. If the rate of encystment equaled the rate of division, then the cell numbers would increase only linearly. In addition, the outer edge of the *N. lovaniensis* growth front was observed to migrate faster than that of isolate 5334. Thus, part of the problem with the isolation of pathogenic *Acanthamoeba* spp. may have been due to the overgrowth of these amoebae by *Naegleria* spp.

After relative growth rates and competition between *N. fowleri* and *N. lovaniensis* were understood, the methodology for environmental screening of pathogenic amoebae was improved. By harvesting cells soon after the environmental sample was planted and then replating these cells over the total surfaces of new plates, an increase in cell numbers could be obtained without a selective loss of *N. fowleri*. Previously, the

TABLE 5. Flow cytometric analysis of environmental samples incubated at 28°C

Sample site (isolate no.)	Flow cytometric analysis				
	Anti-*A. castellanii* antiserum[a]	Anti-*A. culbertsoni* antiserum	Anti-*A. royreba* antiserum	*Acanthamoeba* sp. isolate 5334	Anti-*N. gruberi* antiserum
Flow Stream 6 (1)	−	−	−	−	−
Flow Stream 6 (2)	+			−	−
Flow Stream 6 (4)	+	−	−	−	+
Flow Stream 6 (5)	−	−	−	−	−

[a] +, Cytogram pattern indicative of species; −, cytogram pattern not similar to species.

replanted samples had been placed in the centers of *E. coli* plates, consequently facilitating loss of the pathogen. This development is especially important if the pathogen is in low numbers or if quantitative environmental analysis of *N. fowleri* is desired.

The cell surface fluorescence-to-area ratio, when displayed on the multichannel distribution analyzer, is indicative of the relative number of binding sites for a fluorochrome on the cell membrane (15, 17). Thus, the use of antisera made against the membranes of amoebae, as described by Stevens et al. (21), in conjunction with flow cytometry may, in the future, add to this immunotaxonomic system by quantitatively analyzing the relative binding sites on the membrane for immunoglobulins. Preliminary studies with such antisera (kindly supplied by E. Willaert, Veterans Administration Hospital, Gainesville, Fla.) support this contention (unpublished data).

The ultimate application of the techniques developed in this study is their use in conjunction with a cell sorter for the analysis and isolation of pathogenic and nonpathogenic amoebae from environmental samples. A cell sorter is a modification of a cytofluorograph that not only differentiates subpopulations of cells but also isolates specific subpopulations for further analysis. This would be an invaluable aid in accurately assessing the prevalence of pathogenic free-living amoebae, particularly *Acanthamoeba* spp., in various environments.

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LITERATURE CITED

1. **Anderson, K., and A. Jamieson.** 1972. Primary amoebic meningoencephalitis. Lancet **ii**:379.
2. **Bhagwandeen, S. B., R. F. Carter, K. G. Naik, and D. Levitt.** 1975. A case of *Hartmannellid* amoebic meningoencephalitis in Zambia. Am. J. Clin. Pathol. **63**:483–492.
3. **Carter, R. F.** 1968. Primary amoebic meningoencephalitis: clinical, pathological and epidemiological features of six fatal cases. J. Pathol. Bacteriol. **96**:1–25.
4. **Cerva, L.** 1967. Immunological studies on *Hartmannellid* amoeba. Folia Parasitol. (Prague) **14**:19–25.
5. **Chang, S. L.** 1958. Cultural, cytological and ecological observations on the amoeba stage of *Naegleria gruberi*. J. Gen. Microbiol. **18**:565–578.
6. **Chowdbury, T. C., and A. K. Weiss.** 1975. Concanavalin A, p. 221–241. Plenum Publishing Corp., New York.
7. **Coulson, P. B., and R. L. Tyndall.** 1978. Quantitation by flow microfluorometry of total cellular DNA in *Acanthamoeba*. J. Histochem. Cytochem. **26**:713–718.
8. **Culbertson, C. G., J. W. Smith, and J. R. Minner.** 1958. *Acanthamoeba*: observations on animal pathogenicity. Science **127**:1506.
9. **De Jonckheere, J., and H. Van de Voorde.** 1977. Comparative study of six strains of *Naegleria* with special reference to nonpathogenic variants of *Naegleria fowleri*. J. Protozool. **24**:304–309.
10. **Duma, R. J.** 1972. Primary amoebic meningoencephalitis. Crit. Rev. Clin. Lab. Sci. **3**:163–192.
11. **Duma, R. J.** 1978. The epidemiology of naturally acquired human, waterborne meningoencephalitis due to thermophilic, pathogenic *Naegleria*, p. 107–115. *In* R. M. Gerhold (ed.), Proceedings of the symposium microbiology of power plant thermal effluents. University of Iowa Press, Iowa City.
12. **Fliermans, C. B., R. L. Tyndall, E. L. Domingue, and E. J. P. Willaert.** 1979. Isolation of *Naegleria fowleri* from artificially heated water. J. Therm. Biol. **4**:303–305.
13. **Griffin, J. L.** 1972. Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. Science **178**:869–870.
14. **Jager, B., and W. Stamm.** 1972. Brain abscess caused by free-living amoeba probably of the genus Hartmannella in a patient with Hodgkins disease. Lancet **ii**:1343–1345.
15. **Leary, J. A., and P. Todd.** 1977. Lasar cytophotometric detection of variations in spatial distribution of concanavalin-A cell surface reception following viral infection. J. Histochem. Cytochem. **25**:908–912.
16. **Martinez, J., R. J. Duma, and S. Markowitz.** 1975. Experimental *Acanthamoeba* pneumonitis and encephalitis in mice. Invasion of the central nervous system by *Acanthamoeba*: pathogenesis and ultrastructural features. J. Infect. Dis. **131**:692–699.
17. **Melamed, M. R., P. F. Mulaney, and M. M. Mendelsohn.** 1980. Flow cytometry and sorting, p. 1–40. John Wiley & Sons, Inc., New York.
18. **Mitcheson, J. M.** 1971. The biology of the cell cycle, p. 223. Cambridge University Press, Cambridge, England.
19. **Nagington, J., P. Watson, T. Playfair, J. McGill, B. Jones, and A. Steele.** 1974. Amoebic infection of the eye. Lancet **ii**:1537–1540.
20. **Singh, B. N.** 1975. Pathogenic and nonpathogenic amoebae. Macmillan Publishing Co., New York.
21. **Stevens, A. R., T. Kilpatrick, E. Willaert, and A. Capron.** 1977. Serologic analyses of cell-surface antigens of *Acanthamoeba* spp. with plasma membrane antiserum. J. Protozool. **24**:316–324.
22. **Stevens, A. R., R. L. Tyndall, C. C. Coutant, and E. Willaert.** 1977. Isolation of the etiological agent of primary

amoebic meningoencephalitis from artificially heated waters. Appl. Environ. Microbiol. **34**:701–705.

23. **Tyndall, R. L., E. Willaert, A. R. Stevens, and C. C. Coutant.** 1978. Isolation of pathogenic *Naegleria* from artificially heated waters, p. 117–123. *In* R. M. Gerhold (ed.), Proceedings of the symposium microbiology of power plant effluents. University of Iowa Press, Iowa City.
24. **Tyndall, R. L., E. Willaert, A. R. Stevens, and A. Nicholson.** 1979. Pathogenic and enzymatic characteristics of *Acanthamoeba* isolated from cultured tumor cells. Protistologica **15**:17–22.
25. **Visvesvara, G., and W. Balamuth.** 1975. Comparative studies on related free-living and pathogenic amoeba with special reference to *Acanthamoeba*. J. Protozool. **22**:245–256.
26. **Warhurst, D. C., W. P. Stamm, and E. A. Phillips.** 1976. *Acanthamoeba* from a new case of corneal ulcer. Trans. R. Soc. Trop. Med. Hyg. **70**:279.
27. **Weller, T. H., and A. H. Coon.** 1954. Fluorescent antibody studies with agents of Varicella and Herpes Zoster propagated *in vitro*. Proc. Soc. Exp. Biol. Med. **86**:789–794.
28. **Wellings, F. M., P. T. Amuso, S. L. Chang, and A. L. Lewis.** 1977. Isolation and identification of pathogenic *Naegleria* from Florida lakes. Appl. Environ. Microbiol. **34**:661–667.
29. **Willaert, E.** 1971. Isolement et culture in vitro des amibes du genre *Naegleria*. Ann. Soc. Belge Med. Trop. **51**:701–708.
30. **Willaert, E.** 1975. Primary amoebic meningoencephalitis: a selected bibliography and tabular survey of cases. Ann. Soc. Belge Med. Trop. **54**:205–216.
31. **Willaert, E., A. Jamieson, J. B. Jadin, and K. Anderson.** 1974. Epidemiological and immunoelectrophoretic studies on human and environmental strains of *Naegleria fowleri*. Ann. Soc. Belge Med. Trop. **54**:333–342.
32. **Willaert, E., and A. R. Stevens.** 1976. Indirect immunofluorescent identification of *Acanthamoeba* causing meningoencephalitis. Pathol. Biol. **24**:545–547.