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Agglutination of *Naegleria fowleri* and *Naegleria gruberi* by Antibodies in Human Serum

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The capability of serum samples from 423 human subjects to agglutinate rounded cells of *Naegleria fowleri* nN68 was assessed. Sera from the umbilical cords of seven infants failed to agglutinate *N. fowleri* cells. The median agglutination titer was 1:4 for sera from children through age 4 years, 1:8 for sera from juveniles 5 to 15 years of age, and 1:16 for sera from subjects 15 to 30 years old. The agglutination titers of sera from older adults decreased to a median of 1:8 for the 40- to 60-year-old age group and to 1:4 for the 60- to 90-year-old subjects. Serum samples from young adults agglutinated rounded cells of both *N. fowleri* and *N. gruberi*. The agglutination activity for *N. fowleri* was removed by absorption with *N. fowleri* but not with *N. gruberi*. Conversely, agglutination activity for *N. gruberi* was removed by absorption with *N. gruberi* but not with *N. fowleri*. The agglutinating activity for *N. fowleri* was immunoglobulin M. Serum samples from children displayed markedly disparate capabilities to agglutinate *N. fowleri* and *N. gruberi*. Only rounded cells of *N. fowleri* or *N. gruberi* were reliably agglutinated by human serum samples. Live or paraformaldehyde-killed cells could be used in the assay, but live *N. gruberi* cells returned to the amoeboid form, and these agglutinated poorly.

Primary amoebic meningoencephalitis is a rare, acute, usually fatal disease of active juveniles (4). The disease has been encountered throughout the world, and the etiologic agent, *Naegleria fowleri*, has been isolated from a variety of aquatic environments in both hemispheres (7, 15). Antibodies reacting with *N. fowleri* antigens have been detected in pooled sera from young adult women in the United States (16) and in serum samples from adults and infants in New Zealand (5). Agglutinating activity specific for *N. fowleri* has also been measured in serum samples from 255 human subjects in Richmond, Va. (12). The agglutinating activity for *N. fowleri* could not be absorbed by *N. gruberi*. Sera from human infants had negligible capability to agglutinate *N. fowleri*. By 4 years of age, all children tested had sera with measurable agglutinating activity for *N. fowleri*. Agglutinating activity for *N. fowleri* rose progressively, reaching a maximum at young adulthood.

It had been observed in the previous surveys that *N. fowleri* KUL was not agglutinated as effectively by human sera (12) or hyperimmune mouse sera (9) as other tested strains. This discrepancy could be due to inherent antigenic differences or to physiological heterogeneity. We had noted that strain KUL remained more amoeboid in the diluent used for agglutination assays than most other strains. Accordingly, we compared the capability of human serum to agglutinate rounded cells and amoeboid cells of *N. fowleri* and *N. gruberi*.

One of the objectives of this study has been to gain insight into the source of antigen eliciting the antibodies reacting with *N. fowleri*. Cursons et al. (5) proposed that antibodies to *Naegleria* spp. might have been elicited by exposure to ubiquitous nonpathogenic amoebae such as *N. gruberi*. Our earlier survey, however, failed to detect agglutinating activity for *N. gruberi* in human serum (12). Accordingly, further studies have been carried out to determine whether the inability to detect agglutinating activity for *N. gruberi* reflected limitations of the assay procedure or an absence of specific antibody. Our earlier survey focused on pediatric outpatients and young adults (20 to 40 years old). If continued exposure to a ubiquitous antigen were responsible for the immune response to *Naegleria* spp., older adults should have a level of agglutinating activity similar to young adults. If the antigen were limited to novel sites in the environment (e.g., recreational lakes), more sedentary, older adults should have lower agglutinating activity for *Naegleria* spp. Although fatal

TABLE 1. Capability of human serum samples to agglutinate *N. fowleri* nN68

Age group or source of sera	No. of subjects[a]		No. with agglutinating titers of:													
			<1:2		1:2		1:4		1:8		1:16		1:32		1:64	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Cord sera	4	3	4	3												
1 day to 1 yr	4	4	0	1	4	2	0	1								
1–2 yr	5	6	1	0	2	1	1	4	0	1	1	0				
2–4 yr	4	7			1	4	0	1	2	2	1	0				
4–8 yr	7	3					3	2	3	1	1	0				
8–15 yr	18	12			1	2	2	2	11	4	4	4				
15–30 yr[b]	77	43			1	0	6	3	13	8	30	17	19	8	8	7
40–60 yr	20	34	0	1	1	4	3	7	6	9	7	11	3	2		
60–90 yr																
Institutionalized patients[c]	110		3		20		30		43		14					
Community[c]	62		6		15		25		14		2					

[a] M, Male; F, female.
[b] Previous results (12).
[c] Sex not available.

primary amoebic meningoencephalitis in humans is rare (4), amoebae have also been implicated in an asthmatic-type disease and rheumatoid arthritis (18). A second objective of this study was to test for a correlation between antibody to *Naegleria* spp. and rheumatoid arthritis or asthma.

The present survey has assessed the capabilities of cord sera and serum samples from elderly humans to agglutinate *Naegleria* cells. These subjects had not been included in our prior study (12), and Cursons et al. (5) have reported that cord serum contains antibodies reacting with *N. fowleri*. The current report also presents data on the agglutinin levels in selected patients, in particular those with rheumatoid arthritis. Wyburn-Mason (18) has proposed that amoebae might initiate the changes of rheumatoid arthritis. The current survey establishes the immunoglobulin class responsible for agglutination of *N. fowleri*. A satisfactory method for measuring agglutinating activity of human serum for *N. gruberi* was developed.

MATERIALS AND METHODS

N. fowleri nN68 and 6088 were grown in Nelson medium (10). *N. gruberi* EGB was grown in Balamuth medium (2) or in a medium composed of 9 parts of Nelson medium and 1 part of Balamuth medium.

Samples of fresh human serum were obtained from the Duke-Watts Family Medicine Center, from Marian Waller, Department of Medicine, Virginia Commonwealth University, or from Professional Medical Laboratories, Petersburg, Va. All sera were collected by centrifugation (1,500 × *g* for 20 min) and stored at −20°C until assayed. Complement was inactivated by heating at 56°C for 30 min.

Amoebae for agglutination assays were harvested from axenic cultures in Nelson medium and suspended in Eagle minimum essential medium (12, 15). The suspension was chilled to 5°C and diluted to give 5×10^5 cells per ml. Prolonged chilling of the cell suspension caused spontaneous agglutination. Alternatively, *Naegleria* cells were killed and fixed by adding paraformaldehyde to a final concentration of 1% (vol/vol). The killed cells were washed three times and suspended in Eagle minimum essential medium. The *Naegleria* cells were mixed with serum dilutions in microtiter plates with flat bottoms (B-D Immuno-diagnostics, Oxnard, Calif.) and then incubated at 37°C for 30 min. Agglutination was scored by examining the amoeba suspensions with an inverted compound light microscope. Agglutination titers were expressed as the greatest serum dilution capable of agglutinating the amoebae (1). Duplicate assays did not vary by more than one twofold dilution.

Anti-human immunoglobulin A (IgA), IgG, and IgM antisera (Cappel Laboratories, Cochranville, Pa.) were used to identify the immunoglobulin class present in fractions after DEAE-cellulose chromatography and were used as reagents to block agglutinating activity of human serum samples. For the latter purpose, 50 μl of a human serum sample and 50 μl of anti-immunoglobulin reagent were mixed and incubated at 37°C for 30 min before assaying for agglutinating activity.

Human serum samples were fractionated by DEAE-cellulose chromatography (11). The protein fractions were recovered by stepwise elution with sodium phosphate buffers: step I, 15 mM, pH 6.3; step II, 40 mM, pH 6.0; step III, 100 mM, pH 5.8; step IV, 300 mM, pH 5.5; and step V, 400 mM, pH 4.3. The protein distribution was monitored with a model UA-5/273 fraction collector (ISCO, Lincoln, Nebr.). The flow rate was 80 to 90 ml/h. Samples corresponding to absorbance peaks were pooled and concentrated by dialysis against polyethylene glycol (Carbowax 20,000; Union Carbide Corp., New York, N.Y.).

Sera and serum samples were treated with 200 mM 2-mercaptoethanol to inactivate IgM (9). The treated samples and their untreated controls were incubated at

TABLE 2. Symptoms of human subjects and capability of their sera to agglutinate *N. fowleri* nN68

Symptom	Subjects		No. with agglutinating titers of:					
	No.	Median age (yr)	<1:2	1:2	1:4	1:8	1:16	1:32
Asthma	22	10	1	2	2	16	1	
Brain involvement	8	4	1	3	1	2	1	
Respiratory distress	3	11		2	1			
Rheumatoid factor positive	25	54		4	8	10	3	
Rheumatoid factor negative	25	58		1	2	8	10	4

37°C for 30 min before agglutinating capability was measured.

Ouchterlony gel diffusion assays were performed on selected sera and serum fractions by the method of Garvey et al. (8).

RESULTS

The agglutinating capability of 77 serum samples from infants, children, and juveniles and of samples from 226 adults against *N. fowleri* nN68 was assessed. The sera of all 15 infants, including 7 cord sera, had negligible agglutination titers against *N. fowleri*. The sera of the 22 1- to 4-year-old children had a median agglutination titer of 1:4. Sera of the 120 adolescents and adults had a median agglutination titer of 1:16. Sera of institutionalized subjects in the age range of 60 to 90 years had a median agglutinating titer of 1:8, whereas those living in the community had a median titer of 1:4 (Table 1).

The agglutinating titers of sera from 25 rheumatoid factor-positive and 25 rheumatoid factor-negative adult arthritis patients were compared. The median titer of the rheumatoid factor-positive sera was 1:8, whereas that for rheumatoid factor-negative patients was 1:16 (Table 2). The median agglutinating titer of sera from 22 pediatric asthmatic outpatients was 1:8.

Selected human sera were examined for their capability to agglutinate *N. fowleri* and *N. gruberi*. As noted before, sera from young adults agglutinated rounded cells of *N. fowleri* effectively. These sera agglutinated amoeboid cells of *N. fowleri* poorly (Table 3). Sera from young adults agglutinated rounded cells of *N. gruberi* but not amoeboid cells. The agglutinating activities of human serum samples were generally congruent for rounded cells of *N. fowleri* and *N. gruberi*. Sera from infants did not agglutinate rounded cells of either species (Table 3). Sera from young adults which had been absorbed with rounded cells or amoebae of *N. fowleri* lost the ability to agglutinate *N. fowleri*. Sera absorbed with *N. fowleri* retained the ability to agglutinate *N. gruberi*. Sera absorbed with rounded cells or amoebae of *N. gruberi* specifically lost the ability to agglutinate *N. gruberi*. Sera absorbed with *N. gruberi* retained the ability to agglutinate *N. fowleri* (Table 4). Sera absorbed with sheep erythrocytes also retained the ability to agglutinate *Naegleria* spp.

Several human sera were fractionated by DEAE-cellulose chromatography. By Ouchterlony assays, we confirmed that the majority of the IgG was in fraction 1 and the majority of the IgM was in fraction 4. Essentially all of the agglutinating activity was in fraction 4 (Fig. 1). Anti-human IgM but not anti-human IgA or anti-human IgG blocked the ability of human sera to agglutinate *N. fowleri* (Table 5). The agglutinating activity of whole human serum and of fraction 4 was inactivated by 2-mercaptoethanol. Sera from elderly subjects who had negligible capability to agglutinate *N. fowleri* did not inhibit the ability of sera of young adults to agglutinate *N. fowleri*.

Selected samples of sera from pediatric outpatients were examined for their capability to agglutinate live or killed rounded cells of *N. fowleri* and *N. gruberi*. The agglutinating activities of the pediatric serum samples measured with killed cells as the assay antigen were essentially the same as those measured with live rounded cells (Table 6). There was, however, no correlation between the agglutinating activity of a pediatric serum sample with respect to *N. fowleri* and that for *N. gruberi*.

TABLE 3. Capability of selected human serum samples to agglutinate rounded cells of *N. fowleri* nN68 and *N. gruberi* EGB

Serum sample	Agglutinating activity with respect to:			
	N. fowleri		*N. gruberi*	
	Rounded cells	Amoebae	Rounded cells	Amoebae
Young adult (29 yr)	1:32	1:8	1:32	1:2
Young adult (23 yr)	1:32	1:4	1:32	1:2
Young adult (24 yr)	1:16	1:2	1:8	1:2
Young adult (28 yr)	1:8	1:2	1:8	1:2
Child (12 yr)	1:8	1:2	1:16	1:2
Child (5 yr)	1:8	1:2	1:8	1:2
Elderly (68 yr)	1:8	1:2	1:8	1:2
Elderly (67 yr)	1:4	1:2	1:4	1:2
Toddler (1.5 yr)	1:8	1:2	1:4	1:2
Umbilical cord	1:2	1:2	1:2	1:2

TABLE 4. Absorption of agglutinating activity of a human serum sample by amoebae or rounded cells of *N. fowleri* nN68 or *N. gruberi* EGB

Serum absorbed with:	Agglutinating activity with respect to:			
	N. fowleri		*N. gruberi*	
	Rounded cells	Amoebae	Rounded cells	Amoebae
Nothing	1:64	1:8	1:32	1:2
N. fowleri amoebae	1:4	1:2	1:32	1:2
N. fowleri rounded cells	1:2	1:2	1:32	1:2
N. gruberi amoebae	1:32	1:2	1:2	1:2
N. gruberi rounded cells	1:32	1:2	1:2	1:2
Sheep erythrocytes	1:32	ND[a]	1:16	ND

[a] ND, Not done.

DISCUSSION

Our results indicate that humans are periodically exposed to *Naegleria* immunogen in sufficient amounts to elicit and sustain a readily measurable immune response. The antibody elicited is of the IgM class, indicating that the exposure is rather low or that the antigen may be a repeating polymer, for example, a polysaccharide or glycolipid. The capability of human serum samples to agglutinate rounded cells but not amoebae of *N. fowleri* or *N. gruberi* is consistent with the proposition that rounded cells display unique antigenic sites on their surfaces. The agglutinating activities are species specific, thereby corroborating the suggestion that the agglutinating activity for *N. fowleri* is not the result of prior exposure to the ubiquitous free-living amoeboflagellate *N. gruberi*. Moreover, the agglutinating activity is not absorbed by sheep erythrocytes, indicating that Forssman antigens are not involved. Accordingly, *N. fowleri* itself may be the immunogen eliciting the *N. fowleri* agglutinins. If this proposal is correct, exposure to *N. fowleri* only rarely leads to primary amoebic meningoencephalitis.

The failure to detect agglutination of *N. gruberi* by human serum in our previous survey (12) reflected the rapid reversion to amoeboid cells that occurred during the assay. Good agglutination of either *N. fowleri* or *N. gruberi* is achieved only with rounded cells. Rounding is evoked by chilling cells grown in a medium of low ionic strength (e.g., Nelson medium). *N. gruberi* grown in Balamuth medium reverts rapidly to the amoeboid form and is not suitable for agglutination assays. Rounded cells may be killed and fixed with paraformaldehyde to ensure that they do not revert to the amoeboid form.

Sera from young adult humans contain specific agglutinating activity for *N. fowleri*, but the agglutinating activity is negligible in infants. The agglutinating activity is an antibody of the IgM class; therefore, it is not passed transplacentally to newborns. Toddlers develop agglutinating activity, indicating that antigenic exposure is associated with exploration of the environment. Agglutinating activity is highest in the sera of young adults and wanes in later life. Although impaired responsiveness to *Naegleria* antigens is a possible explanation, it is more likely that decreased exposure to the antigen is responsible for the decreased level of agglutinating activity in the sera of humans over 50 years of age. The source of this antigen or cross-reacting antigen in nature remains unknown. *N. fowleri* can be isolated from nature, particularly from thermally polluted water (7, 15).

In our previous survey (12), the 38 asthmatic pediatric outpatients appeared to have somewhat higher agglutination titers with respect to *N. fowleri* than nonasthmatic children of similar age (median of 1:16 versus median of 1:8, respectively). In the current series of 22 asthmatic pediatric outpatients, the agglutinating activity for *N. fowleri* was the same as that for other

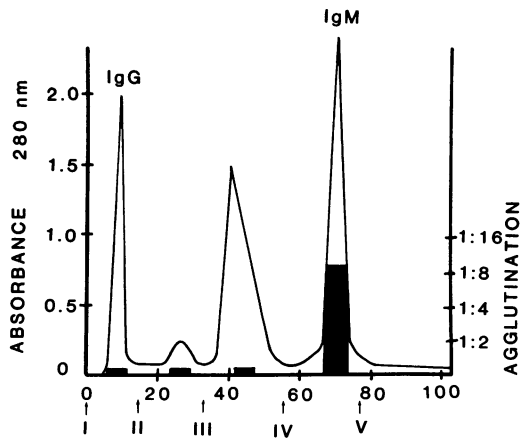

FIG. 1. Capability of fractions of human serum to agglutinate *N. fowleri* nN68. Serum was fractionated by DEAE-cellulose elution chromatography. Protein content of the fractions was monitored by absorbance at 280 nm (———). Agglutinating activities of selected pooled fractions were measured (solid bars).

TABLE 5. Inhibition by anti-human immunoglobulin or by 2-mercaptoethanol of capability of selected human sera to agglutinate *N. fowleri* nN68

Treatment	Agglutinating titer of serum sample			
	1	2	3	4
None	1:16	1:16	1:16	1:32
Anti-IgA	1:16	1:8	1:16	1:16
Anti-IgG	1:16	1:8	1:16	1:16
Anti-IgM	1:4	1:4	1:4	1:4
2-Mercaptoethanol	<1:4	<1:4	<1:4	<1:4
Negative geriatric serum	1:16	1:8	1:16	1:16

children of similar age. The adult patients who were rheumatoid factor positive had lower agglutinating titers for *N. fowleri* than rheumatoid factor-negative subjects of similar age. The lower agglutinating titer is most likely a consequence of the disease state rather than being linked to the causality of the disease.

It has been proposed that unwitting exposure of humans to the more ubiquitous nonpathogenic species may immunize them against the more virulent pathogenic species (1, 6). Our results indicate that the agglutinating activities for *N. fowleri* and *N. gruberi* are distinct and are not likely to represent immunological cross-reactions. Moreover, there is little immunological cross-reaction between *Naegleria* spp. and *Acanthamoeba* (14) or *Entamoeba* spp. (17). We have proposed that juveniles who have not been previously exposed to *N. fowleri* antigens or have not developed antibody against *N. fowleri* or both are vulnerable to primary amoebic meningoencephalitis (12). Cain et al. (3) failed to detect antibody against *N. fowleri* in a patient with primary amoebic meningoencephalitis, using a fluorescent-antibody test. Similarly, Cursons et al. (6) found only a very low intensity of fluorescence, using an indirect fluorescent-antibody test for specific *N. fowleri* antibody, in another patient with primary amoebic meningoencephalitis. The low levels of specific antibodies reported for the patients with primary amoebic meningoencephalitis may accurately reflect the low level present initially or the limitations of the tests used or both. The acute nature of the disease does not usually allow sufficient time to elicit a marked elevation in specific antibodies; however, a titer of 1:4,096 was demonstrated in the serum of a patient successfully treated for primary amoebic meningoencephalitis (13). Data available presently are too limited to allow us to correlate susceptibility to primary amoebic meningoencephalitis with the immune state of a human subject.

TABLE 6. Capability of selected pediatric serum samples to agglutinate live or killed rounded cells of *Naegleria* spp.

Serum sample from:	Agglutinating activity with respect to:			
	N. fowleri 6088		*N. gruberi* EGB	
	Live	Killed	Live	Killed
5-yr-old female	1:64	1:32	1:4	1:4
14-yr-old male	1:32	1:32	1:8	1:4
16-yr-old female	1:32	1:16	1:4	1:4
11-yr-old male	1:16	1:16	1:16	1:16
3-yr-old male	1:8	1:8	1:4	1:4
12-yr-old female	1:4	1:8	1:4	1:4
3-yr-old male	1:4	1:8	1:4	1:8
8-yr-old male	1:4	1:4	1:32	1:32
5-yr-old male	1:2	1:4	1:8	1:4
16-yr-old female	1:16[a]	1:32[a]	1:4	1:4
6-yr-old male	1:16[a]	1:32[a]	1:2	1:4
11-yr-old male	1:16[a]	1:32[a]	1:4	1:8
3-yr-old male	1:2[a]	1:4[a]	1:2	1:4

[a] Agglutinating activity with respect to *N. fowleri* nN68.

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LITERATURE CITED

1. **Anderson, K. G., and A. Jamieson.** 1972. Agglutination test for the investigation of the genus *Naegleria*. Pathology **4**:273–278.
2. **Band, R. N., and W. Balamuth.** 1974. Hemin replaces serum as a growth requirement for *Naegleria*. Appl. Microbiol. **28**:64–65.
3. **Cain, A. R. R., P. G. Mann, and D. C. Warhurst.** 1979. IgA and primary amoebic meningoencephalitis. Lancet **i**:411.
4. **Carter, R. F.** 1972. Primary amoebic meningoencephalitis. An appraisal of present knowledge. Trans. R. Soc. Trop. Med. Hyg. **66**:193–213.
5. **Cursons, R. T. M., T. J. Brown, E. A. Keys, K. M. Moriarty, and D. Till.** 1980. Immunity to pathogenic free-living amoebae: role of humoral antibody. Infect. Immun. **29**:401–407.
6. **Cursons, R. T. M., E. A. Keys, T. J. Brown, J. Learmouth, C. Campbell, and P. Metcalf.** 1979. IgA and primary amoebic meningoencephalitis. Lancet **i**:223–224.
7. **DeJonckheere, J. G., and H. van de Voorde.** 1977. The distribution of *Naegleria fowleri* in man-made waters. Am. J. Trop. Med. Hyg. **26**:10–16.
8. **Garvey, J. S., N. E. Cremer, and D. H. Sussdorf.** 1977. Gel diffusion, p. 313–321. *In* J. S. Garvey, N. E. Cremer, and D. H. Sussdorf (ed.), Methods in immunology, 3rd ed. W. A. Benjamin Inc., Reading, Mass.
9. **Haggerty, R. M., and D. T. John.** 1982. Serum agglutination and immunoglobulin levels of mice infected with *Naegleria fowleri*. J. Protozool. **29**:117–122.
10. **Haight, J. B., and D. T. John.** 1980. Growth of *Naegleria fowleri* in several axenic media. Folia Parasitol. (Prague) **27**:207–212.
11. **Kim, Y. B., S. G. Bradley, and D. W. Watson.** 1964. Characterization of early 19S and late 7S immunoglobulins in the mouse. J. Immunol. **93**:798–806.
12. **Reilly, M. F., M. K. Bradley, and S. G. Bradley.** 1982. Agglutination of *Naegleria fowleri* by human serum. Proc. Soc. Exp. Biol. Med. **170**:209–212.
13. **Seidel, J. S., P. Harmatz, G. S. Visvesvara, A. Cohen, J.**

Edwards, and J. Turner. 1982. Successful treatment of primary amebic meningoencephalitis. N. Engl. J. Med. **306**:346–348.
14. **Stevens, A. R., T. Kilpatrick, E. Wallaert, and A. Capron.** 1977. Serologic analysis of cell-surface antigens of *Acanthamoeba* spp. with plasma membrane antisera. J. Protozool. **24**:316–324.
15. **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.
16. **Tew, J., J. Burmeister, E. J. Green, S. K. Pflaumer, and J. Goldstein.** 1977. A radioimmunoassay for human antibody specific for microbial antigens. J. Immunol. Methods **14**:231–241.
17. **Visvesvara, G. S., and G. R. Healy.** 1975. Comparative antigenic analysis of pathogenic and free-living *Naegleria* species by gel diffusion and immunoelectrophoresis techniques. Infect. Immun. **11**:95–108.
18. **Wyburn-Mason, R.** 1978. The causation of rheumatoid disease and many human cancers. Iji Publishing Co., Ltd., Tokyo.