

Serum Antibodies to *Balamuthia mandrillaris*, a Free-Living Amoeba Recently Demonstrated to Cause Granulomatous Amoebic Encephalitis

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Free-living amoebae cause three well-defined disease entities: a rapidly fatal primary meningoencephalitis, a chronic granulomatous amoebic encephalitis (GAE), and a chronic amoebic keratitis. GAE occurs in immunocompromised persons. Recently, another type of free-living amoeba, *Balamuthia mandrillaris*, has been shown to cause GAE. The finding that this amoeba has caused infection in some healthy children has raised the possibility that humans may lack immunity to *B. mandrillaris*. Human serum was examined for the presence of surface antibodies specific for this amoeba by immunofluorescence. Sera from adults contained titers of 1/64–1/256 of anti-*B. mandrillaris* antibodies (IgM and IgG classes), which did not cross-react with other amoebae. Cord blood contained very low antibody levels, but levels similar to those in adults were seen in serum of 1- to 5-year-old children.

Since the original postulation by Fowler and Carter in 1965 [1] that free-living amoebae present in soil and water were responsible for a rapidly fatal meningoencephalitis in children in South Australia, there have been worldwide reports of fatal cases of this disease and diseases caused by other types of free-living amoebae. *Naegleria fowleri* was named as the agent responsible for the rapidly fatal primary amoebic meningoencephalitis [2]. *Acanthamoeba* species cause two other well-defined disease entities. They are responsible for the development of a granulomatous amoebic encephalitis (GAE) and a chronic amoebic keratitis [2].

In its life cycle, *N. fowleri* displays cyst, trophozoite, and transient flagellate stages. The persistence of specific stages depends on temperature, nutrients, and salt concentrations in the environment. The trophozoite stage is the infective form of this amoeba, as it is for *Acanthamoeba* species which do not have a flagellate stage. *N. fowleri* and *Acanthamoeba* species are ubiquitous in nature (e.g., *N. fowleri* has been isolated worldwide from a variety of sources, such as public water supplies, swimming pools, freshwater lakes, and thermally polluted waters).

Most cases of *Naegleria* meningoencephalitis involve children with a history of swimming in public pools or freshwater lakes [2]. The organism invades via the olfactory neuroepithelium, directly entering the central nervous system (CNS), where the amoebae cause rapid destruction of the olfactory bulbs and a widespread meningitis and hemorrhagic, necrotizing encephalitis. In contrast, *Acanthamoeba* species usually invade the CNS by a hematogenous spread, producing a GAE. Invasion of the CNS follows the establishment of the organism in primary sites, such as the skin, eyes, and respiratory tract [2]. Patients exhibiting GAE do not have an associated history of swimming, are usually either debilitated and chronically ill or undergoing immunosuppressive therapy, or are patients with AIDS [2]. In contrast, chronic keratitis due to *Acanthamoeba* species occurs in healthy individuals who may have experienced minor trauma to the eye or who wear soft contact lenses [2].

In the community, naturally acquired immunity to *Naegleria* and *Acanthamoeba* species plays an important role in restricting the number of cases of primary amoebic meningoencephalitis and GAE [3]. *Naegleria* species does not spread hematogenously [1, 3] and has a restricted invasion route. In contrast, *Acanthamoeba* species spreads hematogenously, owing mainly to a depressed defense system [2, 3]. A number of the components of the immune system are responsible for the immunity displayed against these organisms. These include the ability of the amoebae to activate complement through the alternative pathway (not requiring antibody), the ability of neutrophils and macrophages to kill the amoebae, and the presence of antibodies against these amoebae [4–8].

Another free-living amoeba, *Balamuthia mandrillaris*, was discovered recently and has been shown to cause a fatal GAE [2, 9]. Of interest, many cases previously diagnosed as GAE have now been retrospectively characterized as granulomatous *Balamuthia* encephalitis [2]. Although it has been assumed that

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Balamuthia species, like *Acanthamoeba* species, infect only immunocompromised persons (including AIDS patients), infection with this amoeba of 2 US children [10] and 2 Australian children [11, 12] possessing apparently normal immunity indicates that *Balamuthia* species may be more virulent than *Acanthamoeba* species.

The question of virulence is a crucial one since infections caused by *B. mandrillaris* are believed to also occur in healthy children; thus, this organism is of great concern to our community. Not only do we not know the environmental distribution of the organism, but very little is known about natural host defenses against the amoeba. Knowledge as to whether we possess "natural" immunity to *Balamuthia* species will be of major importance in the education of parents and the community regarding the dangers of this amoeba.

Materials and Methods

Serum samples were randomly collected from healthy adult volunteers. In addition, serum samples were available from 1- to 5-year-old children who had undergone testing for immunoglobulin deficiency and were found to be normal. These subjects were all South Australian residents. Umbilical cord blood was collected as the source of neonatal serum.

B. mandrillaris (leptomyxida ameba: CDC, V039), which was isolated from the brain of a mandrill baboon [9], was obtained from F. L. Schuster (Brooklyn College, Brooklyn, NY) [13]. This species has been well characterized, grown axenically, and used to raise antisera for diagnosing human cases of *B. mandrillaris* meningoencephalitis [9]. The amoeba was cultured axenically according to the method of Schuster and Visvesvara [13]. In brief, *B. mandrillaris* was maintained in growth medium containing biosate peptone (Becton Dickinson Microbiology Systems, Cockeysville, MD), yeast extract (Sigma, St. Louis), *Torula* yeast RNA (Sigma), and ox liver extract (Oxoid, Hampshire, UK) supplemented with 10% fetal calf serum for 5–7 days. When cultures reached confluence, they were chilled on ice for 5–10 min to detach the amoebae. After three washes with Hanks' balanced salt solution (HBSS), the amoebae were fixed with 1% formaldehyde and stored at 4°C for use in experiments. *N. fowleri* and *Acanthamoeba culbertsoni* were similarly cultured in Fulton's medium A supplemented with 10% fetal calf serum and trypticase soy broth as described previously [4, 5].

For immunofluorescence staining to detect anti-*Balamuthia* antibodies, the amoebae were incubated with serum at different dilutions for 1 h at 4°C, as indicated in the results [14, 15]. The cells were washed, and 50 μ L of fluorescein isothiocyanate (FITC)-labeled anti-human immunoglobulin (IgG+IgA+IgM) antisera (1/20 dilution; Behring, Marburg, Germany) was added. To examine for antibody class, we added 10 μ L of FITC-labeled anti-human IgM monoclonal antibodies (PharMingen, San Diego) or 10 μ L of phycoerythrin-labeled anti-human IgG monoclonal antibodies (PharMingen) for 1 h at 4°C. After incubation, the cells were washed and fixed with 1% formaldehyde-Isoton II (Coulter

Electronics, New South Wales, Australia) solution. Anti-amoeba antibodies were quantified by flow cytometry (FACScan with Lysis II software; Becton Dickinson Immunocytometry Systems, Mountain View, CA). In addition, the cell preparations were added to microscope slides in liquid phase and examined by use of a fluorescence microscope.

Results

Human serum contained anti-*Balamuthia* antibodies, as determined by the indirect immunofluorescence test. When the amoebae were viewed with a microscope under UV light, intense surface staining was evident (data not presented). Initial studies demonstrated that the amount of fluorescence could be quantitated by flow cytometry analysis. Flow cytometry of 50 serum samples from adults showed that all contained anti-*Balamuthia* antibodies but with different levels of activity (data not presented). The titer ranged from 1/64–1/256, and the antibodies were IgM and IgG types. Placental transfer of anti-*Balamuthia* antibodies was evident from the presence of these antibodies in cord blood (figure 1). However, the levels were much lower than those in adult serum (titer of \leq 1/4; figure 1). The anti-*Balamuthia* antibody levels in sera from the group of 1- to 5-year-old children were similar to those in sera from the adults (figure 1).

To determine whether the antibodies against *B. mandrillaris* were specific for this amoeba or cross-reactive with *Naegleria* or *Acanthamoeba* species, we examined the effects of serum adsorption with either of these amoeba on the serum titer of the anti-*Balamuthia* antibodies. *B. mandrillaris* (1 mL) was mixed with either *N. fowleri* (2×10^7), *A. culbertsoni* (2×10^7), or HBSS at 4°C for 4 h, and the amoebae were removed by centrifugation. This adsorbed serum was then tested for the presence of antibodies to the 3 types of amoebae. Serum adsorbed with either *N. fowleri* or *A. culbertsoni* retained amounts of anti-*Balamuthia* antibodies equivalent to those of the non-adsorbed serum (table 1); however, the adsorbed serum had lost >98% of the antibodies to either *Naegleria* or *Acanthamoeba* species (table 1). When human serum was adsorbed with 2×10^7 *B. mandrillaris* (at one-half dilution), 89% of the anti-*B. mandrillaris* antibody activity was lost. This showed that the antibodies binding to *B. mandrillaris* were specific for this amoeba.

Discussion

The ability of amoebae that freely live in soil and fresh water to cause disease in humans is of major concern to the community, particularly as there is no effective treatment of CNS infection caused by these organisms, and infection is invariably fatal. We now present evidence that human serum contains antibodies to another type of amoeba, *B. mandrillaris*, that was

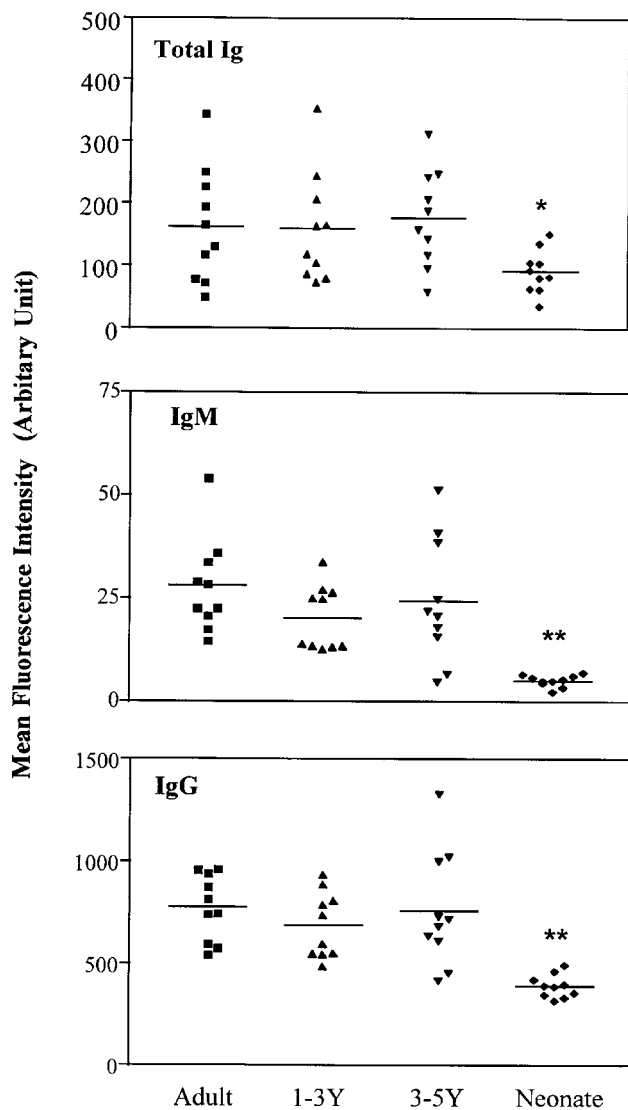


Figure 1. Levels of antibodies to *Balamuthia mandrillaris* in serum from adults, neonates, 1- to 3-year-old children (1-3Y), and 3- to 5-year-old children (3-5Y) as total immunoglobulin (Ig) and IgG and IgM classes. Serum was analyzed by indirect immunofluorescence technique, using flow cytometry at one-fourth serum dilution. Levels for adults and neonates were compared by use of Wilcoxon's signed rank test: * $P < .05$; ** $P < .01$.

recently shown to be responsible for a GAE. This amoeba is believed to invade the CNS secondary to the colonization of primary sites, such as the skin, and invasion of the CNS follows the hematogenous spread of the amoeba. In many cases, successful invasion has been associated with immunosuppression in the infected individual. Previous studies with *N. fowleri* and *Acanthamoeba* species have shown that immunity to these species results from the collaborative action of phagocytic cells and antibody [3]. It is likely that the antibodies to *B. mandril-*

laris also play an opsonization role. Preliminary studies have shown that unlike *Naegleria* and *Acanthamoeba* species, *B. mandrillaris* was not killed (lysed) by human serum, although this serum did promote adherence of neutrophils to the amoeba. It would be interesting to compare the ability of sera to promote neutrophil-mediated killing of the amoeba.

Any invasion from primary sites probably requires some depression of immunoglobulin levels or decreased phagocytic cell function (or both). The reasons why young, healthy children contracted *Balamuthia* infections that developed into a GAE remain unclear [10-12], but in one case, the child exhibited facial lesions that were colonized by the organism [12]. An examination of the serum of these patients for levels of anti-*Balamuthia* antibodies might provide some insight. However, initial studies with serum from our patient [11] who died of *B. mandrillaris* GAE showed an increase in antibody titer of 1/1024 (IgM = 1/64; IgG = 1/1024). This is a 4-fold increase in antibody titer relative to the titer found in control children of similar age, suggesting that the child responded to the amoeba antigens. Whether this represents a protective response or not remains to be established. The rise in the titer, which is well above the range in noninfected children, could provide a means of diagnosing the disease.

Similar to the previous findings with *Acanthamoeba* and *Naegleria* species, anti-*Balamuthia* antibodies were present in the IgM and IgG classes [15]. The serum was also found to be opsonic in attracting neutrophils to bind to *B. mandrillaris* and killing the amoeba (data not presented); this finding could be explored as another marker of immunocompetence. Of interest, the antibodies to this amoeba were specific because they could be adsorbed by *B. mandrillaris* but not *Naegleria* or *Acanthamoeba* species. Since the antibody levels were very low in neonates, the findings suggest that these levels substantially increase with age, probably as a result of environmental exposure to the amoeba in the soil.

The present studies were done with a *B. mandrillaris* isolate that was recovered from a baboon that died from GAE [9]. The isolate is the most characterized isolate, which led to the discovery that it causes human GAE and can successfully be cultured axenically. Indeed, antisera raised against this isolate have been used to diagnose human cases of *B. mandrillaris* GAE

Table 1. Serum anti-*Balamuthia* antibody levels after adsorption with other pathogenic free-living amoebae.

Serum treatment ^a	Antibody titers to		
	<i>B. mandrillaris</i>	<i>N. fowleri</i>	<i>A. culbertsoni</i>
Nonadsorbed	1/256	1/64	1/256
<i>A. culbertsoni</i> -adsorbed	1/256	ND	<1/4
<i>N. fowleri</i> -adsorbed	1/256	<1/4	ND

NOTE. ND = not done.

^a Serum was adsorbed with *Acanthamoeba culbertsoni* or *Naegleria fowleri* and then tested for antibody levels to *Balamuthia mandrillaris*, *A. culbertsoni*, and *N. fowleri* by indirect immunofluorescence assay and flow cytometry.

[9]. It is therefore unlikely that our findings are not representative of human isolates. Our studies have also been confined to the sera of residents of South Australia; studies on sera from a wider international community may add further knowledge to our understanding of natural immunity to *B. mandrillaris*.

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