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Increasing Importance of *Balamuthia mandrillaris*

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INTRODUCTION

Balamuthia mandrillaris is an emerging opportunistic protozoan pathogen, a member of the group of free-living amoebae. *Balamuthia mandrillaris* is known to cause serious cutaneous infections and fatal encephalitis involving the central nervous system (CNS), with a case fatality rate of >98% (total estimated number of cases, ~120). Despite such poor prognosis, the pathogenesis and pathophysiology associated with *Balamuthia* amoebic encephalitis (BAE) remain incompletely understood. Current methods of treatment include a combination approach, where a mixture of drugs is administered, and even then the outcome remains extremely poor. There is an urgent need for improved antimicrobial chemotherapy and/or alternative strategies to develop therapeutic interventions. There is also a tremendous need for education (recognition of possible cases earlier in the infection, as well as prevention possibilities). The purpose of this review is to discuss our current understanding of the biology and pathogenic mechanisms of *B. mandrillaris*.

DISCOVERY OF *B. MANDRILLARIS*

Balamuthia mandrillaris was first isolated in 1986, from fragments of the brain tissue of a mandrill baboon (*Papio sphinx*) that died of a neurological disease at the San Diego Zoo Wild Animal Park in California. The pathophysiological examination revealed that the animal died of a necrotizing hemorrhagic encephalitis similar to granulomatous amoebic encephalitis caused by *Acanthamoeba*. Based on light and electron microscopic studies, animal pathogenicity tests, antigenic analyses, and rRNA sequences, a new genus, *Balamuthia*, was created for this amoeba (80, 81). Later, in 1991, *B. mandrillaris* was associated with fatal human infections involving the CNS (3, 74). Since then, more than 100 cases of BAE have been identified. At present, there is only a single species, *Balamuthia mandrillaris*, in this novel genus.

CLASSIFICATION OF *BALAMUTHIA MANDRILLARIS*

When it was initially isolated from a mandrill baboon, *B. mandrillaris* was classified as a leptomyxid amoeba. Lepto-

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myxid amoebae belong to the subclass Lobosea, comprised of amoebae that move using pseudopodia and exhibit characteristics such as limax, plasmodial, reticulated, and polyaxial forms (2). Limax movement suggests sudden cytoplasmic flow, followed by a break where no movement is observed; such movement has commonly been attributed to leptomyxid amoebae (70). Plasmodial forms suggest that the amoeba may possess more than one nucleus, while reticulate forms may resemble a network of fibers. The term "polyaxial" refers to the ability of an amoeba to travel on more than one axis. Members of the leptomyxid amoeba group all have a thick cell wall in the cyst stage. With the observations of similar properties, *B. mandrillaris* was initially thought to be a member of the Leptomyxidae family. Lately, it has been acknowledged that morphology alone is insufficient for the classification of organisms, and rRNA sequencing has been suggested as a more accurate assessment in the classification of amoebae as well as other organisms (2). However, Visvesvara's amoeba continued to be regarded as limacine until Stothard and coworkers determined the gene sequence of the nuclear small ribosomal subunit RNA (73). These findings revealed that *B. mandrillaris* is the closest evolutionary relative of *Acanthamoeba* (73). Later, Amaral-Zettler et al. (2) determined sequences of several leptomyxid amoebae. Phylogenetic analyses supported a relationship between *B. mandrillaris* and *Acanthamoeba* through calculation of the evolutionary distances (2). It was suggested that *B. mandrillaris* should be moved from the Leptomyxidae family to the Acanthamoebidae family. The findings of Amaral-Zettler et al. (2) and Stothard et al. (73) were further supported by Booton et al. (6, 7), who demonstrated rRNA sequence dissimilarity between *B. mandrillaris* and *Acanthamoeba* strains of between 17.9 and 21.1%, further confirming that *Balamuthia* is closely related to *Acanthamoeba*. Based on these studies, it is currently accepted that *B. mandrillaris* is a close relative of *Acanthamoeba* and is placed in the family Acanthamoebidae (6, 7, 73). A complete scheme of *B. mandrillaris* classification is depicted in Fig. 1. Note that sequence variations in the 16S rRNA genes of all *Balamuthia* isolates tested to date range from 0 to 1.8%, and thus all isolates have been placed in a single species, *B. mandrillaris* (6, 7).

ECOLOGICAL DISTRIBUTION

Generally speaking, free-living amoebae are widely distributed in the environment (62, 63). For example, free-living *Acanthamoeba* and *Naegleria* amoebae have been isolated from a variety of environments (reviewed in references 36, 43, and 82). Although *Balamuthia* has been described as a free-living amoeba, there are only two reports of its isolation from the environment (i.e., soil) (15, 62). It is of interest that in one case, the isolation of environmental *B. mandrillaris* was from soil in potted plants (62). Such soil is often enriched organically with additives (chicken manure, earthworm castings, bat guano, etc.), making it a rich environment for bacterial growth and for organisms that feed on bacteria and one another (15, 62). There are two reports of BAE in dogs who swam in pond water, but the isolation of *B. mandrillaris* from water samples remains undetermined (18, 19). Despite limited success, the precise distribution, niche, or preferred food source of *B. mandrillaris* in the environment is not known. There may be several explanations for this, such as *B. mandrillaris* amoebae being less abundant in the environment than other free-living amoebae, being limited to only certain environmental niches, being difficult-to-isolate and slow-growing organisms (i.e., life cycle of 20 to 50 h), or a combination of the above. A complete understanding of the ecology of *B. mandrillaris* should help us to design strategies to develop preventative measures for susceptible hosts.

ISOLATION OF *BALAMUTHIA MANDRILLARIS*

Unlike *Acanthamoeba*, which is widely distributed in the environment and can easily be isolated from a variety of settings, such as soil, water, and even air, *B. mandrillaris* is difficult to isolate and to culture (61). However, *B. mandrillaris* isolation from soil was recently reported (15, 62). In that case, 5 to 15 g of soil was suspended in 5 to 20 ml of sterile distilled water, and a few drops were plated onto a 1.5% nonnutrient agar plate seeded with *Escherichia coli*. Plates were sealed in plastic bags to prevent drying, incubated at room temperature (~20°C), and examined with an inverted microscope at a magnification of ×100. Following the detection of amoebae of approximately 50 μm which displayed irregular branching structures, agar pieces containing such large amoebae were transferred onto nonnutrient agar plates seeded with *E. coli*. This process was repeated 10 to 20 times until the transferred material was free of contaminating fungi and bacteria. During these transfer steps, *B. mandrillaris* fed on other small soil amoebae and remained in the trophozoite form. Amoebae resembling *B. mandrillaris* were transferred to nonnutrient agar plates seeded with *Naegleria* spp. as a food source (instead of *E. coli*). While promoting growth of *B. mandrillaris*, this step inhibited growth of other amoebae that normally feed on bacteria. Finally, scrapings of the plates with *B. mandrillaris*-like amoebae were transferred to tissue culture flasks containing monkey kidney cells as a feeder layer in the presence of penicillin-streptomycin (200 U/ml). The identity of *B. mandrillaris* was confirmed using immunofluorescence staining assays and PCR methods. Once *B. mandrillaris* amoebae are isolated from environmental samples, they can be cultured routinely on mammalian cell cultures as described in the feeding section. It is noteworthy that the overgrowth of fungi and other protists complicates the isolation of *B. mandrillaris* (15, 62). For example, in the aforementioned study, it took 10 to 20 transfer steps in order to separate the amoebae from the contaminating fungi. An additional problem is the long generation time, ranging from 20 to 50 h. The use of *Naegleria gruberi* to serve as an intermediate food source has been shown to help reduce an overwhelming growth of bacteria (62).

AXENIC CULTIVATION

Schuster and Visvesvara (60) established an enriched medium for the axenic cultivation of *B. mandrillaris*, containing Biostate peptone (2 g), yeast extract (2 g), torula yeast RNA (0.5 g), liver digest (5%), 100× minimal essential medium vitamin mixture (5 ml), 100× minimal essential medium nonessential amino acids (5 ml), 1,000× lipid mixture (0.5 ml), glucose (10%), hemin (2 mg/ml), taurine (0.5%), 10× Hank's balanced salts solution (34 ml), and newborn calf serum (10%).

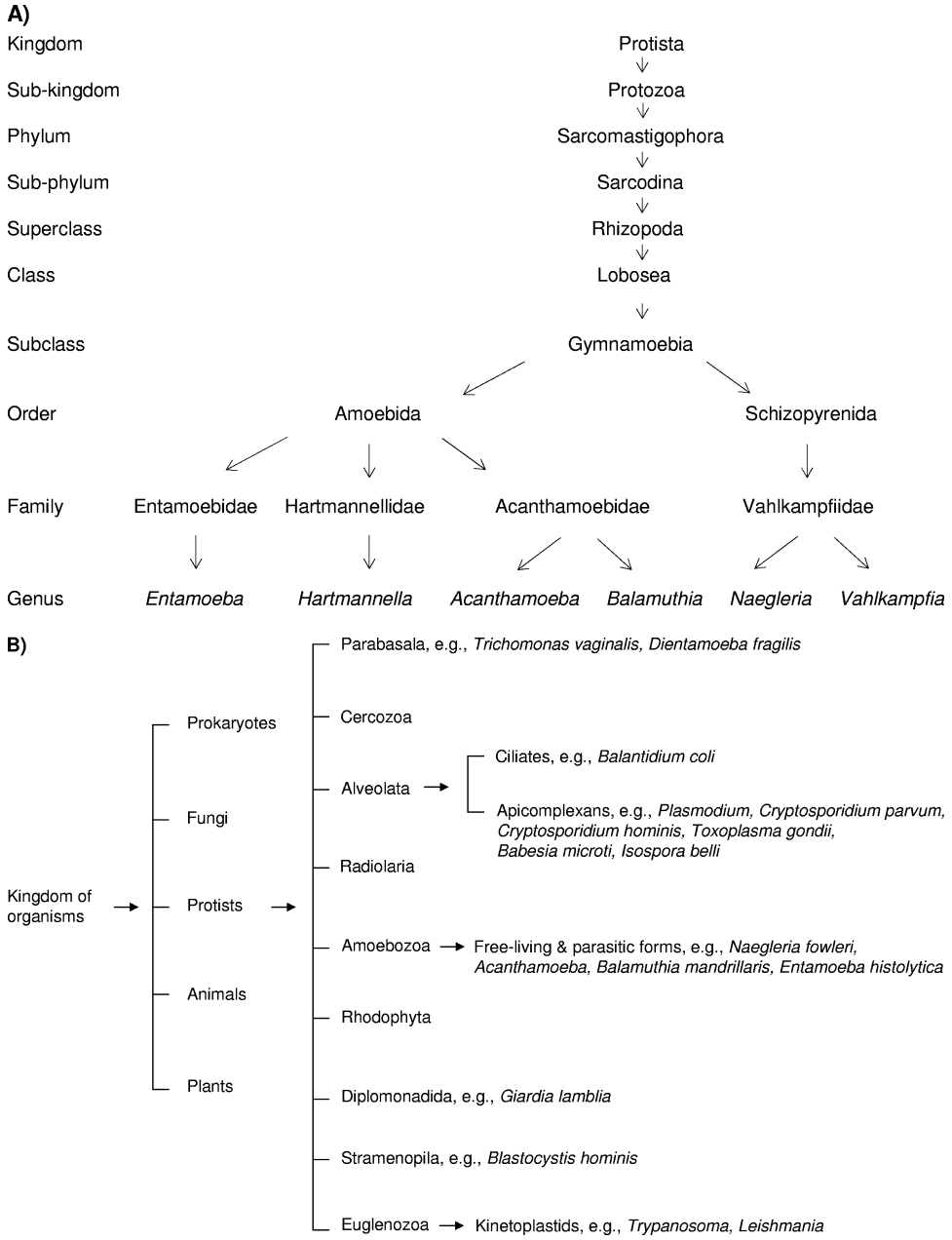

FIG. 1. (A) Traditional classification scheme for free-living amoebae, based largely on morphological characteristics. (B) Present classification scheme for protozoan pathogens, based largely on their genetic relatedness. It is noteworthy that some taxonomists have placed free-living amoebae (*Naegleria* and *Acanthamoeba*) within the kingdom Euglenozoa, based on 18S rRNA sequences.

The pH was adjusted to 7.2 with sterile 1 N NaOH and the final volume made up to 500 ml using distilled water. This medium has been used successfully to culture three strains of *B. mandrillaris*, including the CDC V039 strain, isolated from brain necropsy of a mandrill baboon, and CDC V188 and CDC-V194 isolates from human brains. These amoebae were normally cultured on monolayers of African green monkey kidney cells. Upon axenic cultivation, amoebae grew at various temperatures ranging from 25°C to 37°C (optimal growth at 37°C) and remained viable for up to several months, but they became smaller over time. In contrast, mammalian cultures can be used persistently as feeder cells to culture *B. mandrillaris* amoebae over longer periods, without any modifications in their general appearance.

STORAGE OF *BALAMUTHIA MANDRILLARIS*

Balamuthia mandrillaris trophozoites can be frozen for long-term storage. For optimum revival viability, amoebae are prepared as follows. Briefly, amoebae are suspended in RPMI 1640 and added to tissue culture cells (the food source). When amoebae have cleared the monolayer by ingesting mammalian

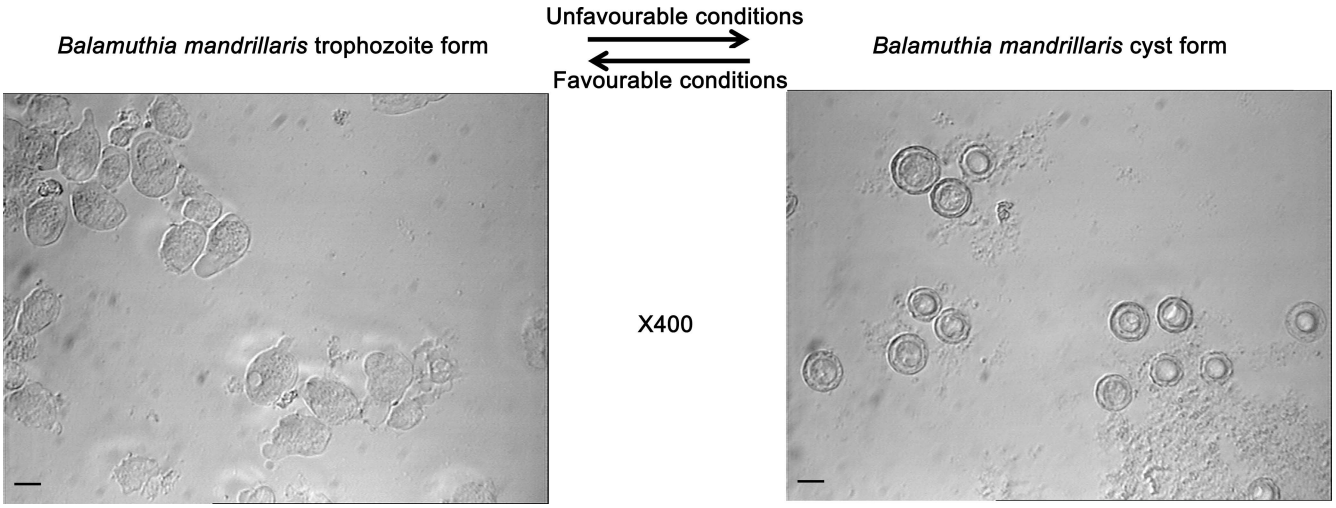

FIG. 2. Life cycle of *Balamuthia mandrillaris*. The infective form of *B. mandrillaris*, also known as trophozoites, exhibits distinct morphological characters under a phase-contrast microscope. Under harsh conditions, trophozoites differentiate into cysts. Although cysts are tripartite (when observed under an electron microscope), under an optical microscope only two layers are observed. Bars, 10 μM.

cell cultures, the flasks are chilled on ice for 5 min to dislodge the amoebae. Amoebae are collected by centrifugation, and the log-phase (actively dividing) amoebae are resuspended at a density of 3×10^6 to 5×10^6 parasites per ml in freezing medium, containing 90% fetal bovine serum and 10% dimethyl sulfoxide. The suspensions are transferred to −20°C for 4 h and then to −80°C for 24 h, followed by storage in liquid nitrogen indefinitely. *Balamuthia mandrillaris* cultures can be revived by thawing at 37°C, followed by their immediate transfer to a mammalian cell monolayer in RPMI 1640 in a T-75 flask at 37°C. In our experience, the aforementioned freezing medium exhibits optimum revival viability.

BIOLOGY AND LIFE CYCLE

The life cycle of *B. mandrillaris* consists of two stages, a vegetative trophozoite stage and a dormant cyst stage (Fig. 2). The trophozoites measure 15 to 60 μm in diameter and have a characteristic irregular branching structure (80). As a eukaryotic cell, *B. mandrillaris* possesses a nucleus but may possess more than one nucleolus (80). Other organelles include mitochondria and the endoplasmic reticulum, containing ribosomes (46, 80). The trophozoite reproduces asexually by binary fission, which is a form of mitosis where the amoeba with its nucleolus and nucleus divides to form daughter cells. Under adverse environmental conditions, such as lack of nutrients, extremes in pH or temperature, overcrowding of cells, or excess of waste products, trophozoites transform into cysts via a process known as encystment (Fig. 2). Encystment ensures the survival of the organism under adverse environmental conditions. Cysts tend to be smaller (i.e., 13 to 30 μm) than the trophozoite stage and are round in structure (Fig. 3). Transmission electron microscopic studies have shown that cysts of *B. mandrillaris* have three walls, with a thin, irregular outer wall known as the ectocyst, a fibrillar middle layer known as the mesocyst, and a thick inner wall called the endocyst (Fig. 3) (81). Under favorable conditions, i.e., availability of nutrients, neutral pH, and moderate temperature (30 to 37°C), cysts differentiate into trophozoite forms via a process known as excystment.

FEEDING (PROKARYOTES, SINGLE-CELL EUKARYOTIC ORGANISMS, AND MAMMALIAN CELLS)

Due to our incomplete understanding of the ecological distribution of *B. mandrillaris*, the preferential food source for this organism remains unclear. Since it is a member of the free-living amoebae, it was thought that *B. mandrillaris* would feed on widely distributed prokaryotes. However, neither gram-negative nor gram-positive bacteria supported *B. mandrillaris* growth (48, 60), even though *B. mandrillaris* incubated with bacteria remained in the active trophozoite stage for more than 10 days, while amoebae incubated alone differentiated into cysts within 24 h. It was further shown that *B. mandrillaris* took up bacteria, as demonstrated using fluorescein isothiocyanate-labeled bacteria. Overall, these studies have demonstrated that *B. mandrillaris* is unwilling to consume bacteria for growth but somehow utilizes bacteria to remain in the trophozoite form. Regardless, it is accepted that *B. mandrillaris* is a free-living amoeba that is distributed in the natural environment. In support of this, it has been shown that the healthy human population possesses antibodies against this potential pathogen (29), suggesting that people normally come across this pathogen in the environment (29). At least one explanation for the occurrence of *B. mandrillaris* in the environment is its ability to feed on other protozoa (most likely amoebae) that are also widely distributed in the environment (reviewed in reference 63). Recent studies examined the use of *Acanthamoeba* as a potential food source for *B. mandrillaris*. It was observed that although *B. mandrillaris* exhibited growth on *Acanthamoeba* trophozoites, it showed a limited ability to target *Acanthamoeba* cysts. This is an interesting finding and may aid in determining the food selectivity of *B. mandrillaris*. For example, *Acanthamoeba* cysts contain cellulose, which is absent in *Acanthamoeba* trophozoites (52). Thus, the differential anal-

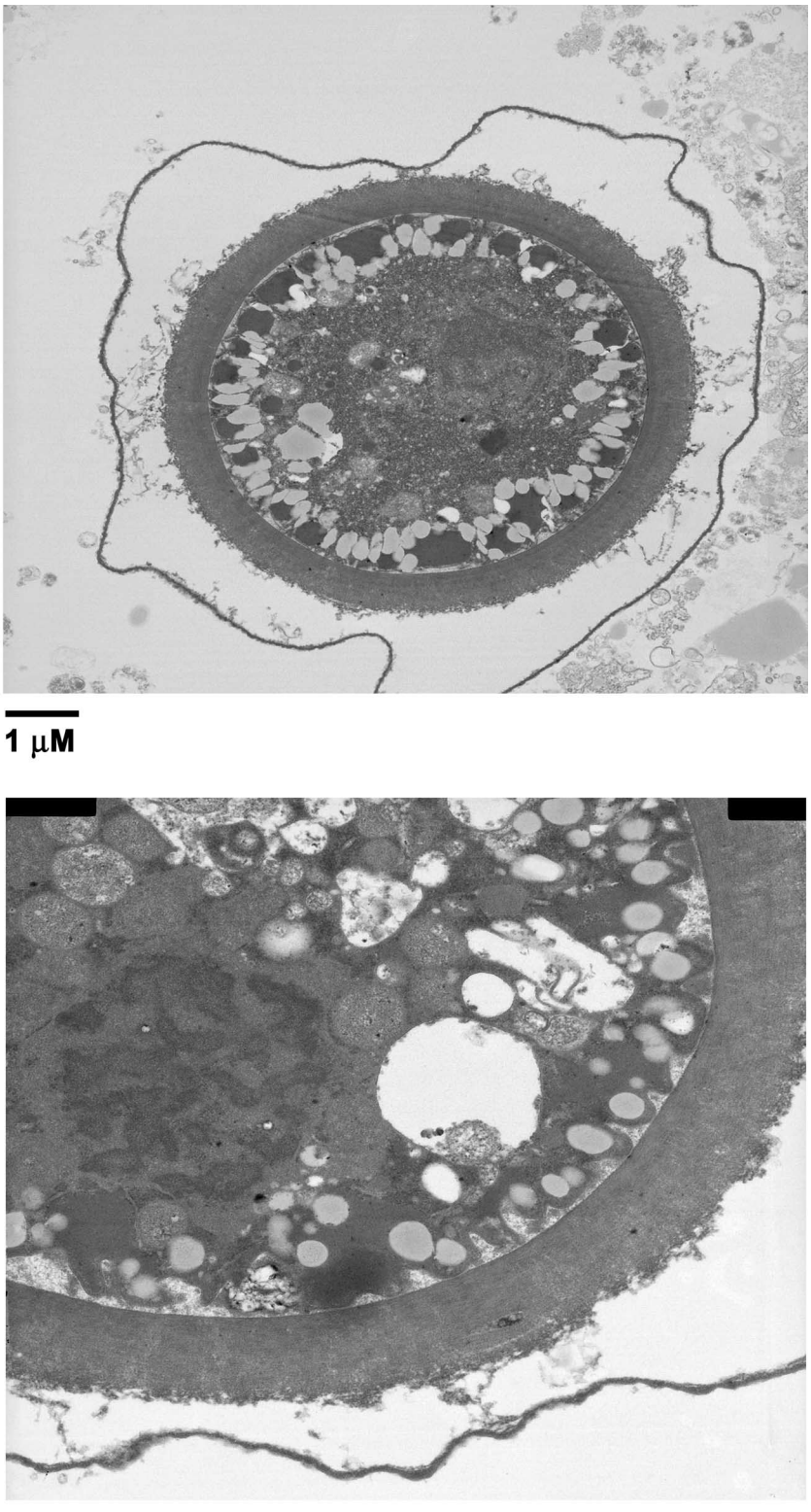

FIG. 3. Transmission electron micrographs of a *Balamuthia mandrillaris* cyst. The wall is made up of a thin, wavy ectocyst, a fibrous mesocyst, and a thick, round endocyst. The cytoplasm is filled with numerous pinocytotic vacuoles and/or vesicles as well as mitochondria. In the lower panel, chromosomes are seen in the nucleoplasm.

ysis of *Acanthamoeba* trophozoites and cysts may provide clues to identify the basis of discriminatory feeding behavior of *B. mandrillaris*. In contrast, as described in the isolation section, *B. mandrillaris* flourishes on mammalian cell cultures. All tested cell cultures, including human brain microvascular endothelial cells (HBMEC), human lung fibroblasts, monkey kidney (E6) cells, and African green monkey fibroblast-like kidney (Cos-7) cells, supported the growth of *B. mandrillaris* (48, 62).

BAE

Epidemiology and Predisposing Factors

BAE is a chronic disease lasting 3 months to 2 years and almost always proves fatal. Unlike *Acanthamoeba* encephalitis, BAE has been found in immunocompetent individuals (individuals who were negative for syphilis, diabetes mellitus, malignancies, and fungal, human immunodeficiency virus type 1 [HIV-1], HIV-2, and mycobacterial infections and had normal CD4- and CD8-postive T-lymphocyte counts and B-lymphocyte counts), which indicates the virulent nature of this pathogen (10, 27, 82). However, the low frequency of BAE suggests the presence of a predisposing factor(s). Whether the predisposing factor is another primary infection, an underlying genetic factor, mere exposure to an environment with widely distributed *B. mandrillaris*, or a combination of the above is incompletely understood. Contact with contaminated soil has been a major risk factor in contracting BAE (15, 62). In one case, a Californian man working in his backyard developed an infection soon after sustaining a puncture wound that was probably contaminated by soil (10), and in a second case, a woman from New York was reported to have worked in her garden with compost soil prior to developing an infection; both of these patients were immunocompetent (34). Furthermore, the majority of cases have been reported from the warmer regions (63), with a significant number occurring in individuals of Hispanic origin (64). Whether individuals of Hispanic origin are more exposed to *B. mandrillaris* (due to socioeconomic status and/or the fact that the majority of the work force in agriculture in America is of Hispanic origin) or whether they have a genetic predisposition to succumb to this disease, as suggested by Schuster et al. (64), remains undetermined and is a key question for future studies. Notably, BAE has been reported for immunocompetent individuals, but patients suffering from diabetes or cancer, HIV-infected patients, drug and alcohol abusers, and patients undergoing steroid treatment, radiotherapy, excessive antimicrobial chemotherapy, or organ transplantation appear to be more susceptible (63). There is a predominance of cases in the young (under 15 years of age) and the elderly (over 60 years of age), which may be attributed to somewhat weaker immune systems. To date, more than 100 worldwide cases have been reported (63). The majority of cases, i.e., 34 documented cases, have been reported from Latin America. Of these, BAE has been recorded in Peru (24 cases), Argentina (4 cases), Brazil (1 case), Mexico (4 cases), and Venezuela (1 case) (20, 44, 57). The total number of reported cases in the United States is approximately 30. The majority of cases in the United States have been highlighted in the Southwest, with California, Texas, and Arizona recording the largest numbers of cases (11, 14, 24, 27, 35). In addition, BAE has been reported in Asia, from Japan (two cases) (68), Thailand (one case) (30), and Australia (eight cases) (55); and in Europe, from the Czech Republic (one case) (37), Portugal (one case) (75), and the United Kingdom (one case) (83). An estimated 25 cases of BAE have been reported for previously healthy, immunocompetent individuals (5, 20, 24, 27, 30, 32, 34, 40, 44, 57, 83). The exact number of cases of BAE worldwide may never be known, which may be attributed to a lack of awareness, poor diagnosis, and poor public health systems, especially in less developed countries. Combinations of drugs are being used for treatment, with very limited success, and this is of growing concern. A summary of the features of BAE is shown in Table 1. BAE is not restricted only to humans but has also been noted in other mammals, including mandrill baboons, monkeys, gibbons, gorillas, sheep, dogs, and horses, with similar disease presentations (reviewed in reference 82).

TABLE 1. Characteristics of BAE

Characteristic and comments
Susceptible hosts Immunocompromised hosts, including HIV/AIDS patients or individuals undergoing organ transplantation or steroid treatment, as well as drug and alcohol abusers; immunocompetent hosts usually include young children and older individuals
Symptoms Headache, fever, nausea, mental state abnormalities, irritability, hemiparesis, cranial nerve palsies, hallucinations, photophobia, sleep and speech disturbance, and seizures
Risk factors Breaks in the skin, working with soil without protective clothing
Incubation period Weeks to months
CSF parameters High protein levels, low glucose levels, amoebae rarely found in the CSF
Gross pathology Multiple necrotic lesions in the brain
Histopathology Cysts and trophozoites found in the perivascular spaces, inflammation with or without granulomas
Laboratory diagnosis Wet mounts of CSF may show the presence of amoebae; indirect immunofluorescence assay of tissue sections, using anti-*Balamuthia* sera, is recommended; in addition, inoculating a portion of clinical sample on a mammalian cell monolayer may result in the isolation of *B. mandrillaris*
Prognosis Extremely poor, with a >98% fatality rate

Portal of Entry

Several routes of entry into the CNS have been suggested. These may include amoeba penetration of the olfactory neuroepithelium via the nasal route. However, hematogenous dis-

semination from a primary lung (amoeba entry via the respiratory tract) or skin (amoeba entry through breaks in the skin followed by exposure to contaminated soil, e.g., working in gardens) entry is thought to be more common (23, 44, 46). In support of this hypothesis, Rideout et al. (56) observed a lack of involvement of the olfactory lobes in animals infected with *B. mandrillaris*, suggesting that the route of amoeba entry into the CNS was hematogenous, but the primary focus was not clear. Moreover, *B. mandrillaris* is usually found within localized areas of the brain and clustered around blood vessels (9, 14, 24, 37, 54, 74, 80). Infections have also caused skin lesions, and in a few cases, amoebae have been found in the kidneys, adrenal glands, pancreas, thyroid, and lungs (3, 11, 23, 56, 57). The fact that multiple tissues became affected suggests that hematogenous spread is an important step in BAE. Cutaneous infection may develop at the site of injury. Following the initial injury, it may take up to several months before the infection develops into BAE. Amoeba entry into the CNS most likely occurs at the sites of the blood-brain barrier (BBB). However, recent studies showed that *B. mandrillaris* may enter the CNS through the choroid plexus. This was demonstrated by the isolation of *B. mandrillaris* from the cerebrospinal fluid (CSF) of a patient who died of BAE (32). The identity of *B. mandrillaris* was confirmed using PCR and culturing, i.e., by inoculating a few drops of CSF directly onto HBMEC monolayers grown in T-75 tissue culture flasks. Although it was widely thought that amoebae may exist in the CSF, there had been a lack of evidence. This finding of *B. mandrillaris* in the CSF indicated that amoeba entry into the CNS may have occurred through the highly vascular choroid plexus.

Clinical Manifestations

Following contraction of *B. mandrillaris* infection, patients initially suffer from headache, stiff neck, nausea, and low-grade fever. As the disease progresses, patients become drowsy and show marked changes in behavior, and their speech may become incomprehensible (46). If the route of entry is breaks in the skin, painless nodules may be observed, developing into lesions containing trophozoites while the patient is alive (30, 53, 83). Such lesions indicate a site of entry and are frequently observed in BAE patients. Symptoms may last from several weeks to months. Some patients exhibit hemiparesis and weakness on part of the face or body, as well as limitations in movement. The condition of the patient may further deteriorate, with a lack of response to stimuli together with pulmonary edema or pneumonia, focal seizures, photophobia, and finally, death (5, 46, 63). A summary of the signs, features, and symptoms of *B. mandrillaris* infections is shown in Table 1. Gross pathological features, including lesions, may also develop in the brain, accompanied by swelling (edema), with soft tissue and necrotic features such as hemorrhagic foci (45, 46). Microscopic examination reveals amoebic trophozoites and cysts in perivascular spaces of brain sections and other infected tissues in patients. The infected brain shows characteristic granuloma formations with multinucleated giant cells, trophozoites, and cysts. The granuloma formation is due to the inflammatory response of the host immune system.

Clinical Diagnosis

Because *Acanthamoeba* encephalitis is limited mostly to immunocompromised patients, susceptible hosts can be monitored for early signs of infection. However, the present data indicate that BAE is more difficult to detect, as it is rare and affects immunocompromised as well as immunocompetent individuals. The unpredictable nature of this disease means that BAE is even less likely to be diagnosed in time for medical intervention, and like the case for *Acanthamoeba* encephalitis and primary amoebic meningoencephalitis due to *Naegleria fowleri*, it is essential for BAE to be diagnosed early if it is to be treated successfully. Clinical diagnosis may be made once general symptoms have been exhibited by the patient. Due to the common features with other types of meningoencephalitis, many cases of BAE infection are diagnosed only at autopsy. These infections are also frequently misdiagnosed and confused with brain tumors, abscesses, toxoplasmosis, or cysticercosis (43, 82). Lumbar puncture, which is the extraction of CSF from between the third and fourth vertebrae, is frequently used to determine the involvement of the CNS in infection. In the case of BAE, normal or low glucose levels and slightly to highly elevated protein levels are normally found (>1,000 mg/dl), as well as the presence of white blood cells in the CSF, which indicates the involvement of the CNS (46). In addition, magnetic resonance imaging and computerized tomography (CT) are useful and will exhibit brain lesions (10, 27, 34, 35). Lesions are found in the parietal lobe, anterior lobe, temporal lobe, and cerebellum (11, 27, 30, 34, 35). In one case, there were as many as 50 lesions present in the brain (27). Lesions have been described as calcified and as forming a mass-like structure (27). Biopsy may reveal features such as a necrotic cortex and the ghostly outline of perivascular monocytes (35). Despite these observations, to date, prognosis remains poor, with only three recorded cases of survival (10, 34).

Once a person is suspected of having contracted BAE, laboratory diagnosis is the best way to confirm infection due to *B. mandrillaris*. This can be achieved using immunofluorescence methods and PCR-based assays. Successful laboratory detection of BAE has been described for immunofluorescence techniques (32, 83). In immunofluorescence assays, fluorescence microscopy can be used to detect the presence of antibodies toward a suspected target antigen of the disease-causing agent. A positive response is indicated by the specific excitation and emission of fluorescence. A PCR assay using rRNA sequences was developed for the specific diagnosis of *B. mandrillaris* (7). PCR assays using Chelex to isolate the DNA from *B. mandrillaris* could detect parasites in small cell numbers. This is important, as clinical samples often contain very few amoebae (32). Alternatively, *B. mandrillaris* can be described directly by histological examination, but it requires expert knowledge of the morphological characteristics of *B. mandrillaris*. Of note, Giemsa stain shows both trophozoites and cysts and may be useful for staining wet mounts, while calcofluor white preferentially stains cysts (has a binding affinity for cellulose, chitin, and potentially other glycans), which may aid in identification. Future studies to determine the chemical composition of *B. mandrillaris* cyst walls are under way. For clinical biopsies/necropsies, hematoxylin-eosin stain is useful in distinguishing amoebae from the host tissue. Culture isolation from biopsies

and/or CSF provides confirmatory evidence. If a patient is suspected of having *B. mandrillaris* infection, the CDC [www.cdc.gov; phone: (770) 488–4417; fax: (770) 488–4253; e-mail: gsv1@cdc.gov] should be contacted for immediate consultation.

Prevention and Control

A significant number of BAE infections have been associated with individuals who had contact with soil (30, 34, 62). It has been suggested that immunocompromised people are the most at risk of contracting BAE infection (56), but healthy individuals with no underlying disease may also be at risk of contracting this infection. As described above, people who are agricultural workers or have regular contact with soil may be more susceptible to BAE infection. Once in the CNS, *B. mandrillaris* is difficult to treat and control, unless it is identified at a very early stage of infection. Clearly, prevention of this infection in the first instance is the most effective solution. Certain simple precautionary measures may be taken to prevent *B. mandrillaris* from entering the host. Individuals with skin lesions should wear protective clothing while working with soil. For example, in one case a child was found to have contracted *B. mandrillaris* infection after playing with soil from a potted plant, which may be an additional risk factor (63).

Although *B. mandrillaris* has not as yet been isolated from water, people who are immunocompromised should be warned of the risks of swimming in freshwater streams, rivers, and lakes, not just for *B. mandrillaris* but also for other free-living amoebae.

Antimicrobial Therapy for BAE

There have been only a few cases of successful treatment of BAE, and the case fatality rate is >98% (10, 27, 34). The limited rate of success depends on early diagnosis followed by aggressive treatment. Due to the lack of knowledge regarding BAE treatment, clinicians have used a mixture of antimicrobials for a successful outcome. However, such combinational therapy may have adverse side effects on the patient (20, 63). To date, successful treatment has been achieved only rarely. In one case, combinational therapy of pentamidine (300 mg intravenously once a day), sulfadiazine (1.5 g orally four times a day), fluconazole (400 mg once daily), and clarithromycin (500 mg three times a day) was administered (34). Another successful outcome was observed through treatment using flucytosine (2 g orally four times a day), pentamidine isethionate (4 mg/kg of body weight/day intravenously), sulfadiazine (1.5 g four times a day), and fluconazole (400 mg/day) for 2 weeks, followed by treatment with clarithromycin (500 mg/day) (34). However, the use of pentamidine was discontinued in both cases cited, because of the side effects. In addition, pentamidine had poor penetration into the CNS when it was tested in HIV/AIDS patients (13). The diamidines, with improved ability to cross the BBB and with low toxicity, would be highly desirable in treating amoebic and other CNS infections (8). A major concern in the two cases described above was recurrence of the infection as a result of reactivation of dormant cysts in the brain lesions. For this reason, patients remained on fluconazole plus sulfadiazine or clarithromycin for several years, but there was no evidence of reactivation of disease and treatment was discontinued. Other drugs are currently being tested as a potential means of combating BAE. Preliminary studies indicate miltefosine (hexadecylphosphocholine) as a potential drug for treating BAE. Miltefosine is an alkylphosphocholine drug previously used for treatment against protozoan diseases, such as leishmaniasis, and is suggested to cross the BBB. It is an enzyme inhibitor and is well absorbed in the human body when taken orally. Miltefosine has been tested against clinical isolates of *B. mandrillaris*, using different concentrations of drug, and showed effective amoebacidal and amoebastatic properties in vitro at concentrations of 30 to 40 μM (63). Despite this, the majority of BAE cases have been fatal. Even with antemortem diagnosis of BAE, initiation of effective antimicrobial therapy may come too late to help the patient (5). The use of flucytosine, fluconazole, pentamidine, and clarithromycin at regular or high dosages was ineffective in four Argentinean pediatric BAE cases (20). Future studies should identify novel drugs and/or determine the potential of known compounds with increased BBB permeability in the treatment of BAE.

PATHOGENESIS OF BAE

Pathogenesis of a disease refers to the ability of a parasite to bring about disease. There is a lack of information regarding the pathogenesis of *B. mandrillaris*. In addition, the ability of *B. mandrillaris* to produce encephalitis in immunocompetent individuals as well as those who are immunocompromised is a matter for concern and indicates the virulent nature of this pathogen (5, 10). As indicated earlier, portals of entry possibly include breaks in the skin and/or respiratory tract followed by amoeba invasion of the intravascular space (30, 63). Hematogenous spread is thought to be a key step in BAE, but it is not clear how circulating amoebae cross the BBB to gain entry into the CNS to produce disease (45). In blood, *B. mandrillaris* is subjected to the immune system of the host, which involves leukocytes, macrophages, neutrophils, and the complement pathway. In addition, antibodies against *B. mandrillaris* are present in healthy populations, with levels of up to a 1:256 titer in human sera (29, 65). However, it has been shown that despite the presence of highly efficient immune systems, *B. mandrillaris* can produce infection in immunocompetent individuals. The crossing of the BBB is indeed thought to be a critical step that is required by many CNS pathogens. First observed in 1885, the BBB is a highly selective barrier which restricts the entry of toxins/pathogens into the CNS due to the presence of tight junctions (16, 22). The BBB is thus critical in the pathogenesis of CNS disease when it fails to prevent an invading pathogen through its selective mechanism. The BBB separates the blood from the CNS. Other cells which constitute the BBB are pericytes and astrocytes (5a). Pericytes are nonneural cells that are thought to regulate capillary blood flow. Astrocytes are thought to regulate signaling pathways across the endothelium and have also been linked with increased transendothelial resistance. Pericytes and astrocytes are surrounded by the basal lamina, over which lies the extracellular matrix. In particular, tight junctions are unique in conferring high endothelial resistances of 1,000 to 2,000 Ω per cm^2 in the brain, which are far higher than those of other endothelial

cells, which are usually less than 20 Ω per cm^2. Molecular components associated with the formation of tight junctions include two classes of transmembrane molecules, including occludin and claudins, which interact with their counterparts on the adjacent endothelial cells. The cytoplasmic tails of these proteins interact with the actin cytoskeleton via a number of accessory proteins, including membranes of zonula occluden (ZO) proteins (5a). The tight junctions seal the BBB, making it impermeable to toxins and other large molecules (>700 Da) (26, 84). An in vitro model of the BBB using HBMEC was previously developed (1, 72). These cells are positive for factor VIII-related antigen, carbonic anhydrase IV, and gamma-glutamyl transpeptidase, indicating their brain endothelial origin (72). Thus, they provide a physiologically relevant model with which to study *B. mandrillaris* traversal of the BBB. To this end, HBMEC were used in our laboratory as a model of the BBB, and we studied their interactions with *B. mandrillaris*.

In studies related to many other pathogens causing meningitis or encephalitis, the pathogen must traverse the BBB to produce disease. There may be three mechanisms by which an amoeba crosses the BBB. The first involves receptor-mediated transport, a contact-dependent mechanism, whereby the amoeba adheres to endothelial cells via an adhesin. In related protozoa, for example, *Acanthamoeba*, mannose binding protein is the adhesin involved in interactions with the BBB (5a). The second mechanism may involve a paracellular route, whereby the amoeba traverses the BBB by crossing between endothelial cells at the tight junctions. The third involves direct crossing by producing damage to the endothelium. Given the size of *B. mandrillaris* (15 to 65 μm), its mechanism of crossing over most likely involves penetration of the barrier. A number of events, such as adhesion of amoebae to HBMEC, cell injury, and the inflammatory response, may combine to disrupt the BBB.

Inflammatory Response to *B. mandrillaris*

Recent studies have shown that *B. mandrillaris* induces interleukin-6 (IL-6) production by HBMEC, which may play a role in *B. mandrillaris* traversal of the BBB (33). These findings are significant, in that IL-6 is known to play a definite role in the pathogenesis of a number of CNS infections. For example, IL-6 antibodies have been found to attenuate inflammation in a rat model of bacterial (pneumococcal) meningitis (42). IL-6 exerts its effect by binding to cell surface receptor complexes (17, 28) and increasing permeability of the BBB by modulating adhesion molecule expression (12). Other cytokines, such as tumor necrosis factor alpha, were tested but did not elicit any remarkable findings (S. Jayasekera et al., unpublished findings). The role of phosphatidylinositol 3-kinase (PI3K) in *B. mandrillaris*-mediated IL-6 release in host cells was investigated by using LY294002, an inhibitor of PI3K, which reduced IL-6 release, confirming the role of PI3K. Furthermore, Western blotting assays confirmed the role of Akt, a known downstream effector of PI3K, indicating that phosphorylation of Akt required the activation of PI3K. The use of dominant-negative cells containing a plasmid encoding a mutant regulatory subunit of PI3K confirmed our findings. In this case, HBMEC which expressed dominant-negative PI3K displayed differences in *B. mandrillaris*-mediated IL-6 release compared to cells with the vector alone (pcDNA3), which showed normal levels of IL-6 release. It was concluded that *B. mandrillaris*-mediated IL-6 release in HBMEC was dependent on PI3K activation. Due to possible transcriptional activities, it is possible that NF-κB may play a role in this signaling pathway. Akt has also been linked to the regulation of transcriptional activity of NF-κB by phosphorylating the p65 NF-κB subunit. Future studies are needed to determine the role of PI3K in NF-κB activation and its involvement in IL-6 release in response to *B. mandrillaris*. The HBMEC model may also be used in future studies to investigate other inflammatory processes associated with the *B. mandrillaris*-mediated response in the host. An interesting subject for study would be nitric oxide release, which has been associated with IL-6 release and BBB permeability (39). A full cytokine profile obtained using a gene expression array could determine specific cell signaling pathways involved in the inflammatory response, apoptosis, and/or necrosis.

Balamuthia mandrillaris Adhesion to the BBB

Using HBMEC, which constitute the BBB, it was recently shown that *B. mandrillaris* produces HBMEC cytotoxicity, which may lead to the BBB perturbations (49). However, the underlying molecular mechanisms associated with amoeba traversal of the BBB leading to pathological features remain unclear. Although the successful traversal of *B. mandrillaris* across the BBB may require multiple events, adhesion is a primary step in amoeba transmigration of the HBMEC. Our recent studies showed that *B. mandrillaris* binds to HBMEC in a galactose-inhibitable manner and identified a galactose-binding protein (GBP) of approximately 100 kDa expressed on the surface of *B. mandrillaris* (49). The presence of GBP in *B. mandrillaris* was recently suggested by Rocha-Azevedo et al. (58), who demonstrated that *B. mandrillaris* binds to laminin and that this interaction can be inhibited using exogenous galactose. Our results support these findings and have identified the expression of GBP on the surface membranes of *B. mandrillaris* amoebae. However, given the complexity of host-parasite interactions, it is tempting to speculate that the aforementioned interactions provide only initial attachment, which is most likely followed by closer associations, with more intimate contact of *B. mandrillaris* with HBMEC involving GBP as well as another adhesin(s). Such binding is probably necessary to withstand blood flow as well as for subsequent crossing of the BBB. It is interesting that GBP inhibited *B. mandrillaris*-mediated HBMEC cytotoxicity. Further studies are needed to validate the concept of other determinants in addition to GBP and their roles in *B. mandrillaris*-HBMEC interactions.

Phagocytosis

The fast and efficient killing of host cells by *B. mandrillaris* suggests the involvement of an active killing mechanism rather than metabolic poisoning or nutrient depletion by the amoeba. Binding leads to secondary events, such as interference with host intracellular signaling pathways, toxin secretions, and the ability to phagocytose host cells, ultimately leading to cell death. Our studies suggest that interactions of *B. mandrillaris* with host cells stimulate specific host cell signaling

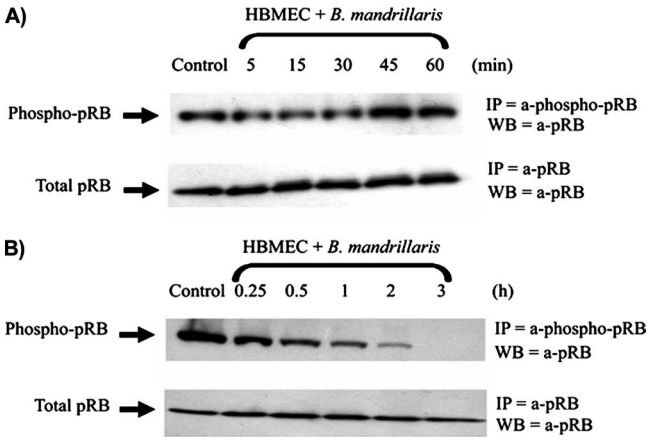

FIG. 4. *Balamuthia mandrillaris*-induced pRB dephosphorylation in HBMEC, using Western blotting (WB) assays. The HBMEC were grown in 60-mm dishes and incubated with *B. mandrillaris* (approximately 2×10^6 amoeba) for up to 60 min (A) or up to 3 h (B). Proteins were immunoprecipitated (IP) with anti-phospho-pRb antibodies and immunoblotted with anti-pRb antibody. In controls, proteins were immunoprecipitated and immunoblotted with anti-pRb antibody. Note that *B. mandrillaris* induced pRb dephosphorylation in HBMEC in a time-dependent manner.

pathways, resulting in host cell death. This was confirmed at the protein level by studying the phosphorylation of the retinoblastoma protein (pRb), a master regulator of the cell cycle (Fig. 4).

Both scanning and transmission electron microscopy revealed extensive morphological changes of the amoebae during feeding, as the target cell is not only enveloped but also penetrated. In axenic cultures, *B. mandrillaris* trophozoites appear in a large variety of shapes, ranging from spherical, almost smooth shapes to highly polymorphic forms with intense surface activity. Both spherical and polymorphic forms can bind to multiple mammalian cells at a time by as yet unknown means (possibly GBP) and then quickly begin to engulf and penetrate their targets, further supporting the concept that target cell lysis and phagocytosis are intimately connected. Amoebastome, which is characteristic of similarly cytopathic *Acanthamoeba* spp., has been observed in *B. mandrillaris* (58).

Ecto-ATPases

Ecto-ATPases are present on the external surfaces of *B. mandrillaris* amoebae (A. Matin et al., unpublished findings). The external localization of the ATP-hydrolyzing site is supported by their sensitivity to suramin, which is a noncompetitive inhibitor of ecto-ATPases and an antagonist of P2 purinoreceptors, which mediate the physiological functions of extracellular ATP. Recent studies have shown, using in vitro assay, that live *B. mandrillaris* amoebae hydrolyze extracellular ATP (Matin et al., unpublished findings). The ability of *B. mandrillaris* to hydrolyze ATP may have a role in the biology and pathogenesis of *B. mandrillaris*. Moreover, by nondenaturing polyacrylamide gel electrophoresis, an ecto-ATPase with a molecular mass of >595 kDa was identified and shown to be heat stable but labile to the detergent sodium dodecyl sulfate. In addition, it was insensitive to ouabain, levamisole, sodium azide, and sodium orthovanadate, further confirming that ATP hydrolysis is due to ecto-ATPase. Previous studies have shown that ADP release from *Acanthamoeba castellanii* plays an important role in its contact-independent cytotoxicity, as demonstrated by increased levels of intracellular calcium, which subsequently lead to apoptosis in Wish cells (51, 69). This is further supported by the inhibition of *Acanthamoeba*-mediated host cell cytotoxicity by use of the ecto-ATPase inhibitor suramin (P2 receptor antagonist), clearly demonstrating that ecto-ATPases play an important role in the pathogenesis of *Acanthamoeba*. Since it is closely related to *Acanthamoeba*, it is reasonable to predict that *B. mandrillaris* may employ similar mechanisms to produce host cell damage. In support of this hypothesis, it was determined that suramin blocked >40% of *B. mandrillaris* binding to host cells (Matin et al., unpublished findings). The role of ecto-ATPase in host-parasite interactions is further supported by the finding that suramin blocks *B. mandrillaris*-mediated HBMEC cytotoxicity.

Considering the fact that *B. mandrillaris* is responsible for brain infection, whose ulceration of the tissues allows the parasite to penetrate into the host CNS, one could speculate that the presence of ecto-ATPase activity might reflect some form of evasion of the parasite from the host defense mechanisms in the circulation. For example, a highly active ecto-ATP-diphosphohydrolase was localized on the external surface of the tegument of *Schistosoma mansoni* (78), and it was suggested that this enzyme might be involved in an escape mechanism allowing the parasite to split ATP released by activated cytotoxic T lymphocytes (78, 79). A similar role may be attributed to *B. mandrillaris* ecto-ATPase; however, this remains to be determined. In conclusion, detection of an ecto-ATPase in *B. mandrillaris* is an important step in understanding the possible role of this enzyme in the pathogenesis of BAE, which may help us to identify potential targets to intervene in this serious infection.

To produce damage to host cells and/or for tissue migration, the majority of pathogens rely upon the ability to produce hydrolytic enzymes. These enzymes may be constitutive enzymes that are required for routine cellular functions or inducible enzymes produced under specific conditions, for example, upon contact with target cells. These enzymes can have devastating effects on host cells by causing membrane dysfunction or physical disruptions. Cell membranes are made of proteins and lipids, and other free-living amoebae, such as *Acanthamoeba*, are known to produce hydrolytic enzymes, such as proteases, which hydrolyze peptide bonds; and phospholipases, which hydrolyze phospholipids (36). Recent studies have shown the presence of phospholipase A, lysophospholipase A, and lipase activities in *B. mandrillaris* (66). However, their specific roles in target cell lysis remain to be established.

In addition, Haider (25) has shown that *B. mandrillaris* exhibits phospholipase A_2 and phospholipase D activities in a spectrophotometric assay. The functional role of phospholipases was determined in in vitro assays by use of HBMEC. It was observed that a phospholipase A_2-specific inhibitor, i.e., cytidine 5′-diphosphocholine, significantly inhibited *B. mandrillaris* binding to HBMEC. Similarly, a phospholipase D inhibitor, i.e., compound 48/80, inhibited *B. mandrillaris* binding to HBMEC. Moreover, both inhibitors partially blocked *B.*

mandrillaris-mediated HBMEC cytotoxicity. Overall, these results clearly demonstrate that phospholipases are important virulence determinants in *B. mandrillaris*.

Proteases

During entry into the CNS from the primary skin lesion or nasal epithelium, amoebae encounter molecules of the host extracellular matrix (ECM), including components of the basal lamina (58). In healthy brains, the ECM comprises a major portion of the normal brain volume (21) that forms the basal lamina around the blood vessels. The ECM is constantly remodeled and provides critical structural and functional support, as well as maintaining homeostasis in the neuronal tissue. However, in neurological disease states, the ECM may undergo substantial modifications resulting in neuroinflammatory responses. The excessive ECM degradation affects neurovascular structural/functional properties that are highly destructive to the CNS functions. The ECM is composed of both collagen types, noncollagenous glycoproteins, and the proteoglycans (41, 59). Thus, the ability of amoebae to degrade the ECM may aid in their invasion of and growth in the brain tissue. Collagen is difficult to degrade due to its helical structure. Recent studies have shown that *B. mandrillaris* exhibits protease properties and is able to cleave type I and III collagen at neutral pH (47), suggesting that *B. mandrillaris* proteases may play a role in ECM destruction. This was further confirmed using a metalloprotease inhibitor, i.e., 1,10-phenanthroline, which completely abolished the protease activities (47). Moreover, *B. mandrillaris* metalloproteases exhibited elastinolytic activities (47). This was demonstrated by the degradation of elastin as a substrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Previous studies have shown that elastase destroys the ECM, which increases BBB permeability, resulting in brain injury (31). For example, injection of elastase into the CSF opened the BBB in newborn piglets (76). In addition to the aforementioned data, the urokinase plasminogen activator system plays an important role in various neuronal diseases involving CNS inflammation and/or pathology. For example, in bacterial meningitis, the urokinase-type plasminogen activator or tissue-type plasminogen activator is known to convert plasminogen (abundant in the brain) (71) into plasmin, which destroys ECM directly by degrading fibrin or by activating matrix metalloproteases. Recent studies have shown that *B. mandrillaris* degrades the proenzyme (47) plasminogen, suggesting that pathogenesis of BAE may involve such pathways. Again, it has been shown that CSF injection of plasmin results in increased capillary permeability in rats (4). Further studies are in progress to determine the activation of plasminogen in response to *B. mandrillaris* metalloproteases and their targets in the neuronal tissue. In tandem, these mechanisms allow *B. mandrillaris* to target ZO-1 and occludin (Fig. 5). As indicated above, both ZO-1 and occludin are involved in the formation of tight junctions, suggesting that *B. mandrillaris* disrupts tight junctions to induce HBMEC permeability and/or disrupts HBMEC monolayers, leading to BBB perturbations and, finally, amoeba entry into the CNS to produce disease.

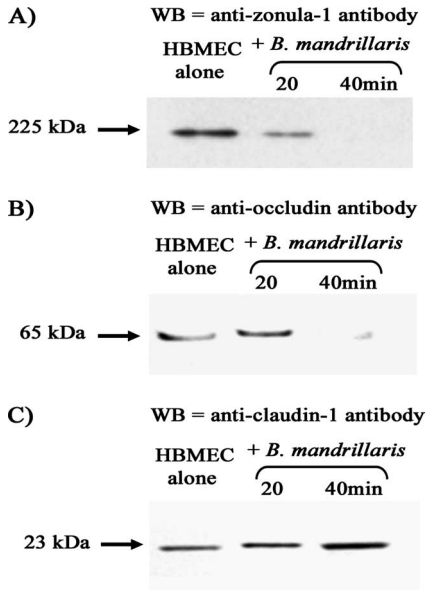

FIG. 5. *Balamuthia mandrillaris*-induced perturbation of the tight junction barrier. *Balamuthia mandrillaris* amoebae were incubated with confluent HBMEC monolayers for various times, and cells were lysed with RIPA lysis buffer. Equal amounts of cell lysates were used for Western blotting (WB) assays using anti-ZO-1 (A), anti-occludin (B), and anti-claudin (C) antibodies. Note that *B. mandrillaris* induced a loss of ZO-1 and occludin within 40 min but had no effect on claudin-1. Results are representative of three independent experiments.

INDIRECT VIRULENCE FACTORS

The ability of *B. mandrillaris* to produce human diseases is a multifactorial process and, among other factors, is dependent on its ability to survive outside its mammalian host for various times and under diverse environmental conditions (high osmolarity, varying temperatures, food deprivation, and treatment with chemotherapeutic drugs). It is most likely the cyst stage that allows *B. mandrillaris* to overcome such conditions. Thus, the ability of *B. mandrillaris* to switch its phenotype can be considered a contributory factor toward disease and can be included among indirect virulence factors.

IMMUNE RESPONSE TO *B. MANDRILLARIS*

Balamuthia mandrillaris antibodies of the immunoglobulin G (IgG) and IgM classes were detected in healthy populations, with titers ranging from 1:64 to 1:256. Cord blood also contained antibodies, but at lower titers, perhaps the result of cross-placental transfer from the maternal circulatory system. However, the antibody levels were very low in neonates, which suggests that these substantially increase with age, probably as a result of environmental exposure to amoebae in the soil (29).

Our recent study showed that serum exhibited protective effects on *B. mandrillaris* binding to and subsequent cytotoxicity of HBMEC (50). Normal human serum exhibited initial limited amoebacidal effects, with approximately 40% of trophozoites being killed. However, a subpopulation of amoebae remained viable, but cultures were stationary over longer incubation times. The fact that serum exhibited approximately

50% inhibition of amoeba binding to HBMEC (similar to amoebacidal effects) suggests that effects of serum on the properties of *B. mandrillaris* are at least partly secondary to the amoebacidal/amoebastatic effects. This is consistent with previous findings, which showed that virulent strains of *Acanthamoeba* resist serum-mediated killing (77). One interesting finding was that serum possesses antibodies that react with several *B. mandrillaris* antigens in Western blot assays (50). The antigens of *B. mandrillaris* reacted strongly with normal human serum. *Balamuthia mandrillaris* strains isolated from baboon tissue (ATCC 50209) and from the human brain shared several common antigens and confirmed that both isolates are antigenically close and belong to the same species. Overall, these studies suggest that normal human serum is partially adept at inhibiting *B. mandrillaris* properties associated with pathogenesis, but whether a healthy immune response is sufficient to control and/or eradicate this life-threatening pathogen is unclear. To this end, studies are being conducted to determine the detrimental effects of serum on *B. mandrillaris* in the presence of neutrophils/macrophages. These studies should clarify the mechanisms associated with *B. mandrillaris* pathogenesis, which may help to design preventative measures and/or develop therapeutic interventions. However, the protective role of antibodies against BAE is somewhat unclear. For example, several BAE patients were reported to possess high titers of anti-*B. mandrillaris* antibodies without a protective response, which resulted in death (32). This may be due to a delayed humoral response, overwhelming BAE infection, or the ability of amoebae to evade the humoral immune response.

BALAMUTHIA MANDRILLARIS AS A HOST

Like other free-living amoebae, such as *Acanthamoeba*, *Naegleria*, and *Hartmannella*, *B. mandrillaris* can act as a host for intracellular survival of bacteria, including the causative agent of Legionnaires' disease, *Legionella pneumophila*. The ability of amoebae to host bacteria enhances bacterial infectivity for mammalian cells, increases their transmission to susceptible hosts, and may enhance the pathogenicity of the host amoeba. Upon incubation with *B. mandrillaris*, *L. pneumophila* remained and multiplied within large vacuoles inside the amoeba. The continued incubation resulted in rounding and detachment of the host amoeba, resulting in its rounding up and complete destruction (67). Overall, these studies suggest that *B. mandrillaris* may harbor pathogenic bacteria in the natural environment and thus may serve as a biological host (i.e., propagation within the amoeba) as well as a transmission vector.

CONCLUSIONS

BAE is a chronic disease that almost always proves fatal. Unlike *Acanthamoeba* granulomatous encephalitis, BAE has been noted in both immunocompetent and immunocompromised individuals. The identification of GBP as a major adhesin for *B. mandrillaris* is important in that it may identify novel targets for the rational development of therapeutic interventions. This is not a novel concept. For example, for *Acanthamoeba*, it was shown that oral immunization with recombinant mannose-binding protein protected animals against *Acanthamoeba* keratitis in vivo. Similar strategies may be developed against BAE, and the identification of GBP should lay a foundation for additional studies. Overall, future research should continue to identify the precise mechanisms associated with the pathogenesis of BAE, as well as host susceptibility, *B. mandrillaris* colonization of skin lesions and/or the nasopharynx, amoeba entry into the intravascular space, parasite survival within the bloodstream, invasion of the CNS, and brain tissue damage leading to encephalitis, which may help to identify potential targets for the rational development of therapeutic interventions and/or design of preventative strategies against BAE.

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