

# Histopathologic spectrum and immunohistochemical diagnosis of amebic meningoencephalitis

Jeannette Guarner<sup>1</sup>, Jeanine Bartlett<sup>1</sup>, Wun-Ju Shieh<sup>1</sup>, Christopher D Paddock<sup>1</sup>, Govinda S Visvesvara<sup>2</sup> and Sherif R Zaki<sup>1</sup>

<sup>1</sup>Infectious Disease Pathology Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA and <sup>2</sup>Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

Traditionally, *Naegleria fowleri* infections are labeled primary amebic meningoencephalitis because of prominent meningeal neutrophilic inflammation. *Acanthamoeba* spp. and *Balamuthia mandrillaris* are labeled granulomatous amebic encephalitis because of parenchymal granulomatous inflammation. We compared histopathologic and immunohistochemical features of 18 cases with central nervous system free-living ameba infections. Immunohistochemical assays using polyclonal antibodies that reacted specifically against each genus identified 11 patients with *Balamuthia* infection, four with *N. fowleri*, and three with *Acanthamoeba*. Immunohistochemical assays highlighted the presence of trophozoites that were difficult to identify with hematoxylin and eosin stains in areas of necrosis or where macrophages were abundant. Immunohistochemical assays also demonstrated the presence of granular antigens inside macrophages and blood vessel walls. Amebic cysts were observed in three patients with *Acanthamoeba* infection and in three with *Balamuthia*. Patients with *Acanthamoeba* infection showed granulomatous inflammation. Patients with *Naegleria* infection had neutrophilic inflammation. *Balamuthia* infections showed a spectrum of inflammation that ranged from primarily neutrophils to granulomas. Meningitis was present in 88% of cases. Immunohistochemical assays were useful to demonstrate the presence of granular antigens and confirmed the genus of the ameba. The spectrum of inflammation in cases of *Balamuthia* meningoencephalitis is broader than previously described. The term amebic meningoencephalitis describes better the histopathologic findings than the currently used classification of primary amebic meningoencephalitis and granulomatous amebic encephalitis.

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*Acanthamoeba*, *Balamuthia*, and *Naegleria* are three genera of free-living amebas that contain species that are pathogenic to humans. *Acanthamoeba* spp. and *Balamuthia mandrillaris* cause subacute or chronic infections of the central nervous system known as granulomatous amebic encephalitis.<sup>1–4</sup> The histopathologic process is characterized by parenchymal necrosis and granulomas, with variations in the amount of inflammation that are dependent on the immunologic status of the host. *Acanthamoeba* and *Balamuthia* trophozoites and cysts are usually found in and around the wall of

small to medium-sized blood vessels causing an angiitis. This angiitis is usually accompanied by hemorrhage, thrombosis, and necrosis. In contrast, the rapidly fatal infection caused by *Naegleria fowleri* causes prominent meningeal inflammation and is known as primary amebic meningoencephalitis. The histopathologic process in cases with primary amebic meningoencephalitis is characterized by a fibrinopurulent leptomeningitis associated with necrosis, hemorrhage, and edema of the brain parenchyma. *N. fowleri* trophozoites are usually observed in the perivascular spaces and cysts are usually absent.<sup>1–4</sup>

Serologic assays have been used for premortem diagnosis of amebic infections with inconsistent success.<sup>5,6</sup> Other diagnostic techniques, including microscopy of wet mounts, histopathology, electron microscopy, immunocytochemistry (such as immunofluorescence, flow cytometry), and culture, rely

Correspondence. Current address: Dr J Guarner, MD, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, 1405 Clifton Road, NE, Atlanta, GA 30322, USA.  
E-mail: Jeanette.Guarner@choa.org  
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on identification of trophozoites and cysts in cerebrospinal fluid or brain tissue specimens.<sup>1,7,8</sup> Previous histopathologic reports describe single cases or small series of patients infected by one amebic genus.<sup>9–20</sup> Side-by-side histopathologic comparison of cases caused by different ameba is not available. To better characterize the central nervous system pathology caused by the three different genera of free-living ameba, we evaluated the histopathologic and immunohistochemical features of patients infected with *Acanthamoeba*, *Balamuthia*, and *Naegleria*.

## Materials and Methods

Immunohistochemical assays for free-living amebas were applied to formalin-fixed, paraffin-embedded tissue specimens from 42 patients with clinically or pathologically suspected amebic central nervous system infections. These samples were sent for consultation to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) from 1998 to 2006. Eighteen patients were included in the study based on morphologic and immunohistochemical evidence of free-living amebic meningoencephalitis. Demographic and clinical data varied in completeness. Hematoxylin and eosin stains were evaluated for the type of inflammation and angiitis as defined in Table 1, and presence of necrosis, hemorrhage, trophozoites, and cysts in the meninges and brain parenchyma.

## Immunohistochemistry

Immunohistochemical assays were performed as previously described for other infectious agents.<sup>21,22</sup> Sections (3  $\mu$ m) of the tissues were placed on

**Table 1** Histopathologic description of angiitis and type of inflammation used in the cases

Feature	Description
Angiitis	Infiltration and damage of a vessel wall by inflammatory cells and protozoa
Acute inflammation	Neutrophils were the most prominent inflammatory cell component present
Chronic inflammation	Lymphocytes and macrophages were the predominant inflammatory component
Mixed inflammation	Equal proportion of neutrophils, lymphocytes, and macrophages was present
Granulomatous inflammation	Presence of foamy macrophages and multinucleated giant cells accompanied by lymphocytes
Mixed granulomatous inflammation	Presence of foamy macrophages and multinucleated giant cells accompanied by neutrophils

Colomark Plus slides (Erie Scientific Co., Portsmouth, NH, USA), deparaffinized, and rehydrated through graded alcohols and distilled water. Tissue sections were washed in Tris–saline–tween 20 buffer (pH 7.5) and placed in a DAKO Autostainer (Dako, Carpinteria, CA, USA). Digestion was performed by soaking slides in 0.1 mg/ml proteinase K (Roche, Indianapolis, IN, USA) in 0.6 Tris–saline–tween/0.1% CaCl<sub>2</sub> (buffer) for 15 min at room temperature, followed by a blocking step with 20% normal sheep serum in Tris–saline–tween. Tissue sections were incubated for 60 min with either polyclonal rabbit anti-*Acanthamoeba castellanii*, *Balamuthia mandrillaris*, or *Naegleria fowleri* antibodies, respectively (Parasitic Diseases Branch, CDC). Optimal dilutions of the antibodies had been determined in previous experiments using formalin-fixed cultures of the three genera of ameba (1:30 000 for the *Acanthamoeba* and *Balamuthia* antibodies and 1:20 000 for the *Naegleria* antibody). After being incubated with the primary antibody, slides were washed, and swine anti-rabbit biotinylated link antibody, alkaline phosphatase-labeled streptavidin, and naphthol fast red chromogenic substrate (LSAB2 Universal alkaline phosphatase system, Dako) were sequentially applied. Sections were then counterstained in Mayer's hematoxylin (Fisher Scientific, Pittsburgh, PA, USA).

Each polyclonal antibody reacted against formalin-fixed, paraffin-embedded cultures of the specific ameba genus it was raised against (for example, the anti-*Acanthamoeba* antibody reacted against *A. culbertsoni* and *A. castellanii*) and did not cross-react with formalin-fixed cultures of the other genera of free-living ameba that were tested. Negative tissue controls consisted of sequential sections incubated with the other anti-ameba antibodies. Interpretation of immunohistochemical results included evaluation of: (1) the location of the positive reaction (ie, necrotic brain parenchyma, meninges, or blood vessels), (2) the parasite staining pattern (trophozoites, cysts, or granular antigen staining), and (3) the strength of the reaction compared to the tissue control.

## Results

Table 2 presents age, sex, duration of illness, and pathologic features of the 18 patients with amebic meningoencephalitis. All patients expired except for two. The first was a 33-year-old male receiving highly active antiretroviral treatment for AIDS with CD4 cell counts of 82/mm<sup>3</sup> who had a discrete brain lesion that showed a granulomatous *Acanthamoeba* infection on a biopsy specimen; he survived 22 months after having been treated with fluconazole and pyrimethamine.<sup>13</sup> The second patient was a 5-year-old female who presented with seizures that were attributed to two distinct lesions in the temporal and parietal lobes; *Balamuthia* infection

**Table 2** Demographic, clinical, and pathologic data of the 18 patients with amebic meningoencephalitis

Origin	Age	Sex	Illness duration	Underlying disease	Genera	Parenchymal inflammation	Meningitis	Trophozoites	Cysts
US	65 y	M	8 d		<i>Naegleria</i>	Acute	Yes	Rare	NI
US	7 y	M	Unknown		<i>Naegleria</i>	Acute	Yes	Rare	NI
US	9 y	M	Unknown		<i>Naegleria</i>	Acute	Yes	Rare	NI
US	12 y	M	3 d		<i>Naegleria</i>	Mixed	Yes	Rare	NI
Spain	33 y	M	1 wk	AIDS	<i>Acanthamoeba</i>	Granulomatous	Yes	Rare	Abundant
US	53 y	M	1 m	Transplant	<i>Acanthamoeba</i>	Granulomatous	Yes	Abundant	Abundant
US	70 y	F	3 m		<i>Acanthamoeba</i>	Mixed granulomatous <sup>a</sup>	Yes	Abundant	Abundant
Peru	14 y	Unknown	2 m		<i>Balamuthia</i>	Acute	NI	Abundant	Abundant
US	31 y	M	3 wk		<i>Balamuthia</i>	Mixed	Yes	Rare	Rare
US	9 m		3 d		<i>Balamuthia</i>	Mixed	Yes	Rare	NI
US	50 y	F	3 m	Rheumatoid arthritis	<i>Balamuthia</i>	Mixed	Yes	Rare	NI
US	5 y	F	3 wk		<i>Balamuthia</i>	Chronic	NI	Rare	NI
US	40 y	M	Unknown		<i>Balamuthia</i>	Mixed granulomatous <sup>b</sup>	NI	Rare	NI
Mexico	11 y	F	7 m		<i>Balamuthia</i>	Mixed granulomatous	Yes	Rare	NI
Mexico	11 y	F	2 m		<i>Balamuthia</i>	Mixed granulomatous	Yes	Rare	NI
Philippines	8 m	F	1 m		<i>Balamuthia</i>	Granulomatous	Yes	Abundant	Rare
US	Unknown	Unknown	Unknown		<i>Balamuthia</i>	Granulomatous	Yes	Rare	NI
US	13 y	F	2 wk		<i>Balamuthia</i>	Granulomatous	Yes	Rare	NI

Abbreviations: d, days; m, months; wk, weeks; NI, not identified; US, United States of America; y, years.

<sup>a</sup>With abundant eosinophils.<sup>b</sup>Forming an abscess.

was diagnosed in a biopsy sample, and she was alive 2 years after having received a variety of treatments including ketoconazole, flucytosine, clarithromycin, and pentamidine.<sup>19</sup>

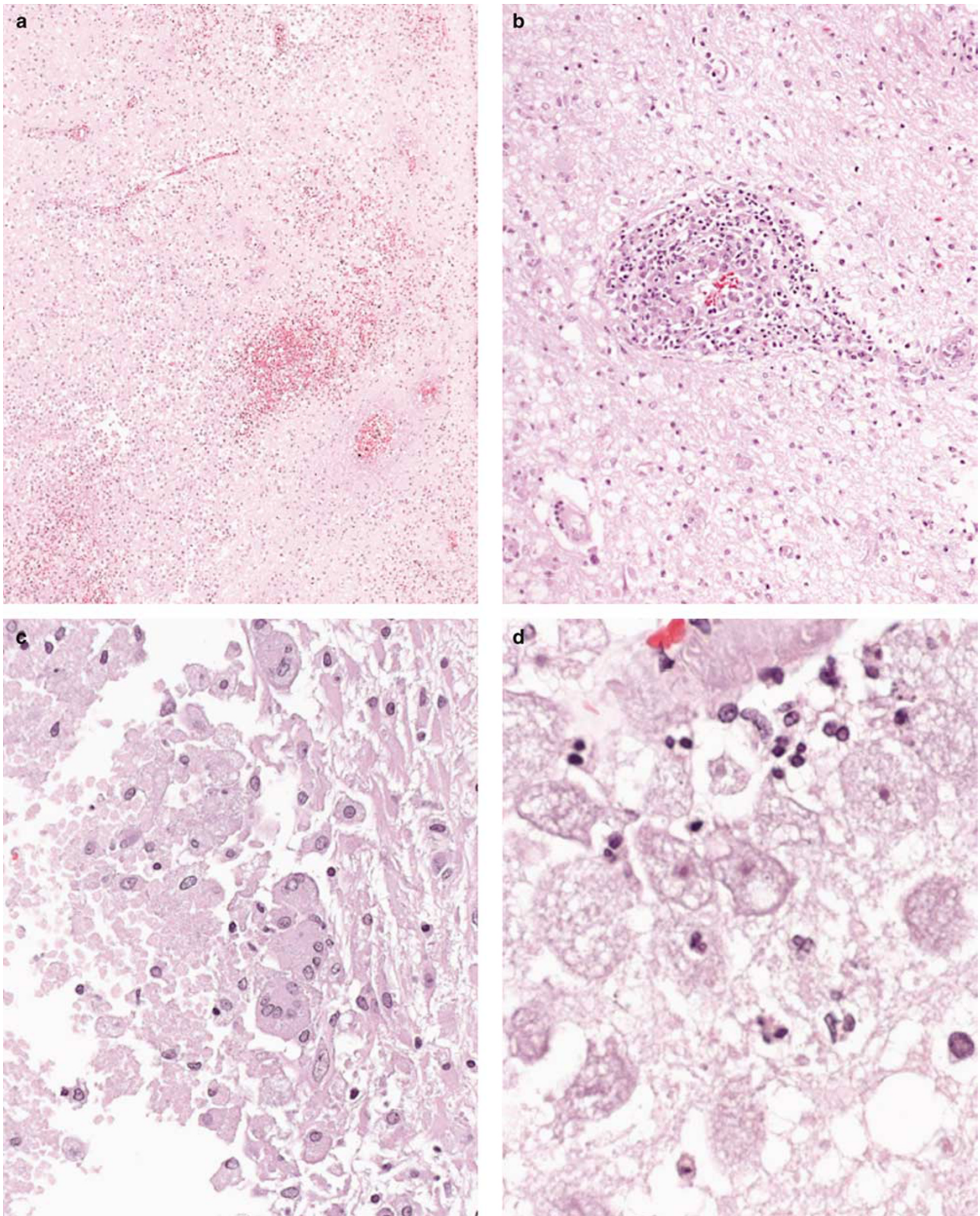
Information regarding the presence of an underlying health problem was available for three patients. A 53-year-old woman who had been treated with a bone marrow transplant for chronic lymphocytic leukemia, a 50-year-old woman with rheumatoid arthritis, and the 33-year-old man with AIDS already described.<sup>13</sup> Information about duration of illness was incomplete but the patients with *N. fowleri* generally died within days following symptom onset, while the duration of symptoms was much broader (ranging from days to months) for patients with *Acanthamoeba* and *Balamuthia* infections.

Parasite morphology and immunohistochemical assays demonstrated the presence of *Balamuthia* in 11 patients, *Naegleria* in four, and *Acanthamoeba* in three patients. All cases showed necrotizing encephalitis (Figure 1a), 14 had angiitis (Figure 1b), and 10 had hemorrhage. The type of inflammation present in the brain parenchyma of each case is presented in Table 2. Patients with *Naegleria* showed predominantly acute inflammation (Figure 1a), *Acanthamoeba* infected patients mostly had granulomatous inflammation (Figure 1c), and those with *Balamuthia* showed a spectrum from acute (Figure 1d) to granulomatous inflammation. Eosinophils were prominent in only one patient with *Acanthamoeba* infection.

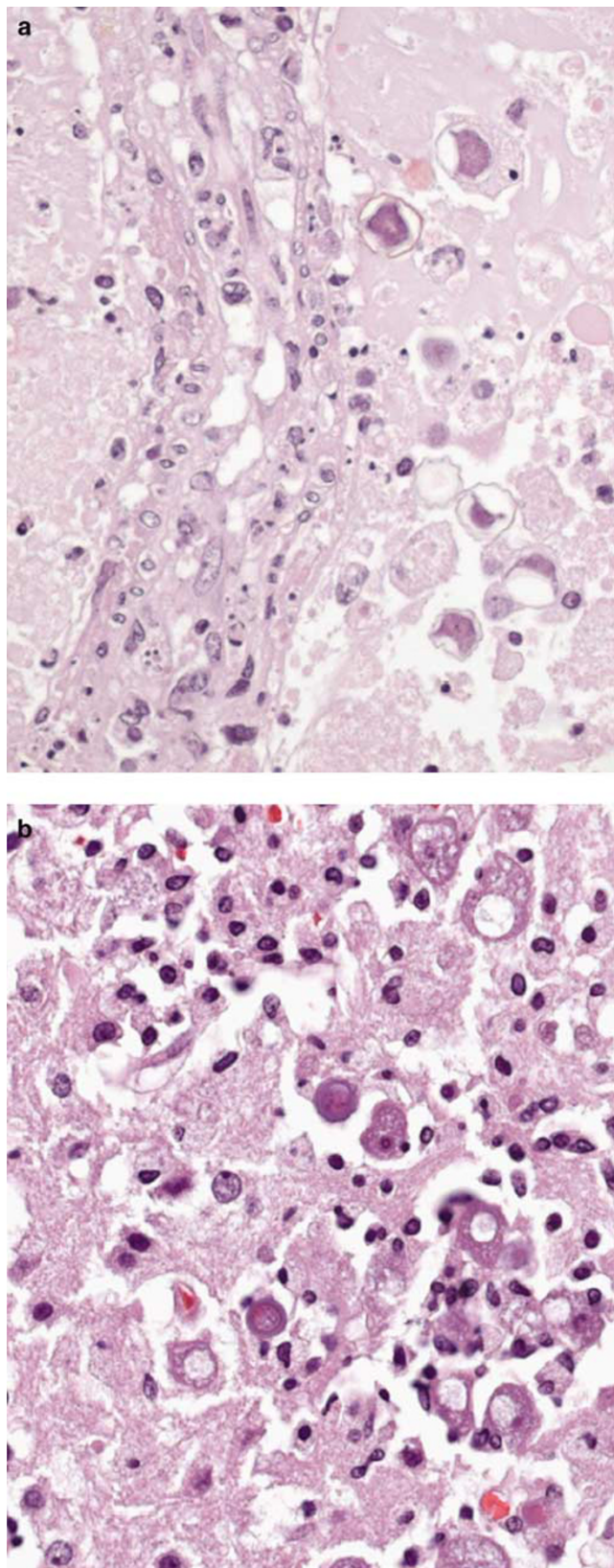
Ameba trophozoites were usually observed around vessels in the brain parenchyma of all cases; however, in most cases, trophozoites proved to be difficult to identify on initial review of hematoxylin and eosin stains because of intense infiltrates by macrophages or presence of necrotic debris. The trophozoites were readily evident with hematoxylin and eosin stains in two patients with *Acanthamoeba* (Figure 2a) infection and two with *Balamuthia* (Figure 2b). Amebic cysts were observed in the three patients with *Acanthamoeba* (Figure 2a) infection and in three with *Balamuthia* (Figure 2b).

Meninges were available for study in 17 cases and inflammation was present in 15 (88%). There was acute meningeal inflammation in the four cases with *Naegleria* infection. Two patients with *Acanthamoeba* showed mixed inflammation and one chronic inflammation in the meninges. Patients with *Balamuthia* infection showed a spectrum of inflammatory meningeal infiltrates including two with mixed inflammation, four with chronic, one with mixed granulomatous and one with granulomatous. Five (28%) cases showed trophozoites in the meninges, two had *Naegleria*, two *Balamuthia*, and one *Acanthamoeba*. None of the cases showed cysts in the meninges.

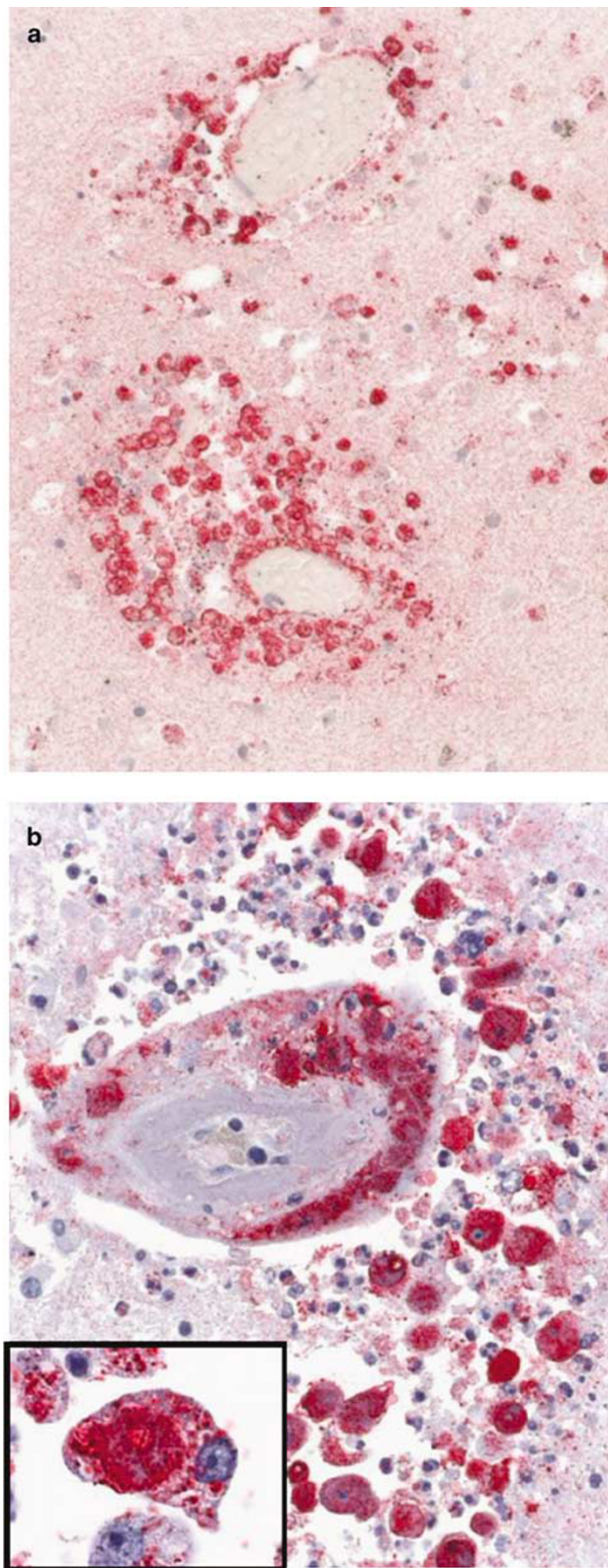
Trophozoites of each species stained strongly with the specific immunohistochemical assay (Figure 3a and b) including those present in areas of necrosis



**Figure 1** Histopathologic features of amebic central nervous system infections showing necrotizing encephalitis (a), angiitis (b), and granulomatous (c) and neutrophilic inflammation (d). Trophozoites are easily identifiable in a patient with *Balamuthia* infection (d). Hematoxylin and eosin stains of infection with *Naegleria* (a), *Balamuthia* (b and d), and *Acanthamoeba* (c). Original magnifications: (a)  $\times 25$ , (b)  $\times 50$ , (c)  $\times 100$ , (d)  $\times 157$ .



**Figure 2** Morphologic characteristics of trophozoites and cysts present in cases with *Acanthamoeba* (a) and *Balamuthia* (b) infections. (Hematoxylin and eosin stains. Original magnifications: (a)  $\times 100$ , (b)  $\times 157$ .)



**Figure 3** Photomicrograph of immunohistochemical assays for *Naegleria* (a) and *Balamuthia* (b) showing brightly stained trophozoites and granular antigens in the blood vessel walls and in macrophages (inset). (Immunohistochemistry using polyclonal antibodies against *Naegleria* and *Balamuthia* and naphthol fast red chromogenic substrate. Original magnifications: (a and b)  $\times 100$ , inset  $\times 250$ .)

(Figure 3a) which had been difficult to identify with hematoxylin and eosin stains because their nuclei were not evident. Cyst walls were faintly stained with the immunohistochemical assays. Immunohistochemical assays demonstrated the presence of granular antigens prominently in vessel walls (Figure 3a and b) particularly for tissues of patients with *Naegleria* and *Balamuthia* infections. Granular antigen fragments were also observed inside macrophages (Figure 3b inset) and endothelial cells. In one *Balamuthia* case, the *Acanthamoeba* antibody faintly stained the ameba, suggesting that there is some crossreactivity of the antibodies. Of the 42 cases tested with the ameba antibodies, 24 tested negative and included two patients with tuberculous meningitis, one with *Streptococcus pneumoniae* meningitis, one with rabies encephalitis, two with infarcts, and 18 with meningoencephalitis for which an infectious agent could not be determined.

## Discussion

This comparative study of cases with central nervous system infections by free-living amebas showed that the current classification of primary amebic meningoencephalitis and granulomatous amebic encephalitis is not as clearcut as previously thought. In our study, *N. fowleri* infections were associated with abundant neutrophilic inflammation in the meninges similar to what has been described in primary amebic meningoencephalitis. *Acanthamoeba* infections showed predominantly granulomatous inflammation in the brain parenchyma corresponding to previously described granulomatous amebic encephalitis.<sup>1–4</sup> However, the histopathology of *Balamuthia* infections showed a wider spectrum of the inflammatory reactions, from acute inflammation only to mixed inflammation with or without granulomas. The variations in the inflammatory reaction observed in cases with *Balamuthia* infections suggest there are cases with intermediate histopathologic features that do not conform to the currently established primary amebic meningoencephalitis and granulomatous amebic encephalitis nomenclature. Shirabe *et al*<sup>11</sup> reported presence of neutrophilic inflammatory infiltrate with small granulomatous lesions in a 78-year old with *B. mandrillaris* meningoencephalitis and Sjogren's syndrome. They postulated that the intermediate pathologic features between primary amebic meningoencephalitis and granulomatous amebic encephalitis were a reflection of the altered immune status of the patient. Documentation of underlying health conditions that could alter the immune status in our patients with *Balamuthia* infections and intermediate histopathologic features was not available. Alternatively, the inflammatory reaction could reflect the duration of illness since *Naegleria* infections have a rapid clinical course and intense neutrophilic inflammation while *Acanthamoeba*

infections have a protracted clinical course and granulomatous inflammation.<sup>2</sup>

We found meningoencephalitis in 88% of the cases regardless of the genera of ameba causing disease. It is postulated that *N. fowleri* enter through the olfactory mucosa causing acute meningitis, while *Acanthamoeba* and *Balamuthia* enter through lungs, skin, or mucous membranes and disseminate to the brain hematogenously causing parenchymal lesions such as abscesses or encephalitis.<sup>15,17</sup> In our series, the meningeal inflammation appeared as a primary component of the disease process in patients with *N. fowleri* infections and prominent acute meningitis and in those with *Balamuthia* infections and granulomatous meningeal inflammation. The prominent meningeal inflammation in some patients with *Balamuthia* infection suggests possible entry of the ameba through olfactory mucosa. Since meningeal inflammation is a frequent occurrence for all free-living amebic infections of the central nervous system, the term meningoencephalitis describes better the histopathologic findings than the currently used classification of primary amebic meningoencephalitis and granulomatous amebic encephalitis.

For diagnosis of amebic infections, it is imperative to document the presence of amebic trophozoites or cysts. In the cerebrospinal fluid, *N. fowleri* trophozoites are frequently found while *Acanthamoeba* and *Balamuthia* are rarely observed.<sup>2</sup> In our series, 50% of cases with *Naegleria* infections showed trophozoites in the meninges, 33% with *Acanthamoeba*, and 18% with *Balamuthia*. Although trophozoites can be abundant and have been described as easily identifiable in tissue samples by using hematoxylin and eosin stains in the literature, in most of our cases the amebas were difficult to identify. The difficulty identifying amebas can relate to the number of parasites and the amount of inflammation and necrotic debris in the tissue section.<sup>20</sup> In addition, differentiation of trophozoites from macrophages requires experience in recognizing the characteristic nuclear morphology of these parasites particularly in areas of intense inflammation and necrosis. An alternative to using specific immunohistochemical assays for amebas can be the use of immunohistochemical assays for macrophages such as CD68; as macrophages are highlighted, nuclear morphology can be studied in the nonstaining mononuclear cells which could correspond to amebas. Grocott's methenamine silver stains *Acanthamoeba* cysts and was useful in our cases to highlight them.<sup>23</sup> However, the silver stains did not contribute further to what was already observed with hematoxylin and eosin stains where the characteristic cysts with wrinkled ectocyst walls were found in cases of *Acanthamoeba* infection.<sup>3</sup> Cysts are not usually found in cases with *N. fowleri* infection because patients typically die before trophozoites can become encysted. In our series, ameba cysts were observed in three cases with

*Acanthamoeba* infection and in three cases with *Balamuthia*. Interestingly, the cases with *Balamuthia* cysts had acute or mixed inflammatory pattern.

Fluorescent assays that use antibodies to detect amebic antigens have been used to highlight the presence of trophozoites. Colorimetric detection of antibodies in an immunohistochemical assay permitted the use of light microscopy and was helpful to better define the interaction of the free-living amoebas in the pathological context.<sup>1,10-17</sup> By using immunohistochemistry, trophozoites were highlighted in areas of necrosis or where there was intense inflammation and distinction between amoebas and macrophages had been problematic with hematoxylin and eosin stains. It was also important in small biopsy specimens of patients who survived where the material examined was scant and consisted primarily of necrotic tissues. In addition, granular antigen staining, representing amoeba fragments and antigens, was observed inside macrophages and endothelial cells, as well as extracellularly in vessel walls. Presence of *Acanthamoeba* antigens has been described in patients with amebic keratitis and has been postulated to account for persistent inflammatory reaction.<sup>24</sup> Granular antigens in parasitic and bacterial diseases may explain continued host reaction to the microorganism and can be useful for diagnosis in the correct clinico-epidemiologic setting.<sup>21,22,25</sup>

Diagnostic techniques that do not rely on visualization of trophozoites, such as polymerase chain reaction, have been developed.<sup>7,26-28</sup> As molecular techniques are being applied more frequently for diagnosis of a variety of infectious agents, false-positive reactions will occur. Thus, molecular results have to be correlated with epidemiologic, clinical, and pathologic features. For example, one of the patients evaluated with the anti-amoeba antibodies had a false-positive polymerase chain reaction for *Naegleria*; however, light microscopy, immunohistochemistry, and fluorescent antibody assays failed to confirm the presence of amoeba or granular antigens but demonstrated viral inclusions that were positive for rabies antigens by immunohistochemistry.<sup>29</sup> This underscores the broad differential diagnosis of amebic meningoencephalitis, which includes bacteria (eg *Mycobacterium tuberculosis*, *S. pneumoniae*), neurotropic viruses, and noninfectious pathologies such as central nervous system infarcts.

In summary, the main histopathologic abnormality in cases with amebic infections of the central nervous system was meningoencephalitis. The spectrum of inflammation in cases of *Balamuthia* meningoencephalitis is broader than previously described. Immunohistochemical assays were useful to confirm the genus and highlight the amoeba, particularly in necrotic areas. Presence of granular amebic antigens in vessel walls and endothelial cells may contribute to angiitis.

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