Neuropathological and Ultrastructural Features of Amebic Encephalitis Caused by *Sappinia diploidea*

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**Abstract.** Here we present the neuropathological, ultrastructural, and radiological features of *Sappinia diploidea*, a newly recognized human pathogen. The patient was a 38-year-old man with visual disturbances, headache, and a seizure. Brain images showed a solitary mass in the posterior left temporal lobe. The mass was composed of necrotizing hemorrhagic inflammation that contained free-living amebae. Immunofluorescence microscopy showed that the organism was not a species of ameba previously known to cause encephalitis. Trophozoites had a highly distinctive double nucleus, and transmission electron microscopy confirmed that they contained 2 nuclei closely apposed along a flattened surface. The 2 nuclei were attached to each other by distinctive connecting perpendicular filaments. This and several other unique structural features led to the diagnosis of *S. diploidea* encephalitis. The patient was treated postoperatively with a sequential regimen of anti-amebic drugs (azithromycin, pentamidine, itraconazole, and flucytosine) and is alive after 5 years. Guidelines to recognize future cases of *S. diploidea* encephalitis are as follows. 1) It presented as a tumor-like cerebral mass without an abscess wall. 2) It had central necrotic and hemorrhagic inflammation that contained acute and chronic inflammatory cells without granulomas or eosinophils. 3) It contained trophozoites (40–70 μm diameter) that contained a distinctive double nucleus. 4) Cyst forms in the host were not excluded or definitely evident. 5) Trophozoites engulfed host blood cells and were stained brightly with Giemsa and periodic acid-Schiff. 6) Trophozoites often were present in viable brain parenchyma on the periphery of the mass without inflammatory response. 7) The prognosis after surgical excision and medical treatment was favorable in this instance.

**Key Words:** Amebic encephalitis; Electron microscopy; Inflammation; Parasite; Trophozoite.

**INTRODUCTION**

Amebic encephalitis is caused by infection of the brain with specific genera of free-living amebae. It has a very low incidence and usually is lethal. Primary amebic encephalitis (PAE) is a rapidly progressive infection by trophozoites of *Naegleria fowleri* (1, 2). The organisms gain access to the brain through nasal mucosa, olfactory nerves, and the cribiform plate, often after the patient swims in freshwater pools. Granulomatous amebic encephalitis (GAE) produces a less fulminant yet lethal infiltration of the brain that affects immunosuppressed and debilitated subjects. GAE is usually caused by cysts and trophozoites of *Acanthamoeba* species (1, 2). *Balamuthia mandrillaris* is a pathogenic ameba first recognized after an outbreak of encephalitis in a mandrill and other primates at the San Diego Wild Animal Park (San Diego, CA) in 1990 (3–5). As in GAE, immunosuppression is a major predisposing factor, including examples in persons with the acquired immunodeficiency syndrome. Focal encephalitic lesions due to *Entamoeba histolytica* (brain abscesses) also can occur in subjects with amebic dysentery (6). We treated a patient with a brain lesion that contained amebae unlike the organisms noted above and we announced the existence of this newly discovered pathogen in a preliminary communication (7). Here, we illustrate fully the histological and highly unique ultrastructural features of the organism that led to its identification as *Sappinia diploidea*, a ubiquitous ameba found in soils and animal feces that heretofore had no medical significance.

**MATERIALS AND METHODS**

**Neurological, Neuroradiological, and Neurosurgical Findings**

A previously healthy 38-year-old white male coastguardsman presented with a history of loss of consciousness for 45 min and emesis, followed by bifrontal headache, photophobia, and blurry vision for 2 to 3 days. Magnetic resonance imaging (MRI) showed a solitary 2-cm mass in the posterior left temporal lobe that showed slight ring enhancement after Gadolinium (Fig. 1). The prior medical history was notable only for a recent frontal sinus infection. Four days later a left temporo-parietal craniotomy and frameless stereotaxy-assisted resection was performed. Cryosections of the specimen examined intraoperatively showed necrotizing hemorrhagic inflammation that contained free-living amebae. Beginning on the first postoperative day, antimicrobial therapy was given based upon potentially effective agents against pathogenic amebae, as follows. Azithromycin 250 mg peroral/d for 31 weeks, intravenous pentamidine 300 mg/d for 6 weeks, itraconazole 200 mg bid, and flucytosine 2.75 gm qid for 25 weeks.
RESULTS
Neuropathology, Immunofluorescence, and Electron Microscopy

The center of the mass contained hemorrhagic necrosis and an angiodestructive inflammatory infiltrate. The inflammatory infiltrate in the regions around necrotic blood vessels contained polymorphonuclear leukocytes (PMNs), hemorrhage, and lymphocytes. More peripheral sectors of the mass contained viable brain tissue that did not have hemorrhagic necrosis; here the inflammatory exudate consisted predominantly of perivascular lymphocytes, macrophages, and very scarce plasma cells. There were no granulomas or eosinophils in the exudate. Free-living amebic trophozoites were clustered around blood vessels and also were observed as single organisms within viable uninflamed brain parenchyma in the peripheral margins of the mass (Fig. 2). Amebic trophozoites stained brightly with Giemsa and periodic acid Schiff (PAS) and were 40 to 70 μm in diameter (Fig. 3). They often contained a single large cytoplasmic vacoule (7), and many trophozoites contained phagocytosed host leukocytes and erythrocytes (Fig. 4). The morphology and size of the trophozoites was similar to other pathogenic amebae, but these organisms had a completely unusual and defining morphological feature, not described previously within any other known human pathogen. Specifically, the trophozoites had a highly distinctive double nucleus in which the 2 nuclei were closely apposed with a central flattening. In thickly sliced frozen sections, 2 nuclei of the double nucleus were stained clearly with hematoxylin and were surrounded by a well-demarcated halo that likely corresponded to nucleoplasm (Fig. 4). Two nuclei were also evident in Giemsa-stained paraffin sections, and the outer nuclear membrane of the double nucleus was sometimes evident (Fig. 5). Paraffin sections of the lesion were sent to the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia for immunofluorescence microscopy. Using a panel of antibodies against amebae recognized to be potentially pathogenic, the results were entirely negative (Table). A fresh sample of tissue was sent to the CDC to isolate an organism. Growth requirements of this organism were unknown and viable amebae were not isolated after extensive effort.

A fresh sample of infected brain tissue was immersed in paraformaldehyde for transmission electron microscopy (TEM), but the sample was necrotic. Another sample was obtained from a block of viable brain tissue that was fixed in buffered formalin and embedded in paraffin. TEM showed that the organisms had typical ameboid shapes and they often displayed a characteristic double nucleus. Two apposed nuclei were flattened at the point of juncture, and depending on the plane of section, both nuclei often contained a dark nucleolus (Fig. 6). The flattened junction point between 2 compressed nuclei showed a striking and completely novel feature. Rows of short filaments perpendicular to the flattened nuclear membranes connected the 2 nuclei (Fig. 7). Less specific features of amebae were present, including cytoplasmic vacuoles, mitochondria with characteristic tubular patterns of cristae (Fig. 8), a juxtanuclear Golgi-like network characteristic of protozoan organisms (Fig. 6), and granular cytoplasmic material. These TEM features confirmed conclusively the hypothesis that a unique ameba was present.

DISCUSSION

The distinctive double nucleus of the amebic trophozoites was the key to recognizing that a unique pathogen was present in this brain lesion. The negative results of comprehensive immunofluorescence studies performed by the CDC supported that impression. The patient was not debilitated or immunosuppressed, which is the main risk for infections with Acanthamoeba and Balamuthia species (1–6). The patient’s favorable clinical outcome was not compatible with Naegleria fowleri infection. There was no apparent infection outside of the brain, as would be expected with brain abscesses due to Entamoeba histolytica. Given the unusual morphological appearance of this pathogenic organism and the patient’s atypical clinical course, 14 postoperative months elapsed before one of the authors (GSV) determined the correct morphological classification of the organism after an exhaustive historical search of protozoan literature.

Specific molecular and immunological tools are not available to assist in identifying S. diploidea in histological sections. Recognizing the pathogen depends primarily on the presence of the distinctive “diploid” nucleus, each containing a nucleolus. Double nucleoli were visualized most easily in cryosections stained with hematoxylin, in part due to their increased thickness relative to paraffin sections. We compared our morphological observations with a detailed light and electron microscopic study of isolated S. diploidea and noted a striking and diagnostic concordance. Goodfellow et al described clearly the unique way that the 2 nuclei are flattened and joined together when observed at the ultrastructural level as follows: “Where nuclei of a pair are flattened against each other, poorly defined fibrils bridge the gap between their membranes” (8). Their illustration of that striking feature matches what was observed in the brain biopsy. The electron density of the membranes does not appear completely identical due to differences in fixation and specimen processing (Fig. 7). The unique configuration of the mitochondrial cristae of S. diploidea we observed also was illustrated in the cultivated organisms (Fig. 8). Goodfellow et al and Noble both described a large contractile vacuole (8, 10), and we also observed that feature in electron micrographs (Fig. 6) and in histological sections using the Giemsa stain (7). The prominent Golgi apparatus we observed also was recognized previously.
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Fig. 2. Inflammation in the brain specimen. The region shown here contains viable brain tissue at the periphery of the mass at right and does not have hemorrhagic necrosis. These areas contained perivascular infiltration of lymphocytes and few or no plasma cells or polymorphonuclear leukocytes, shown at left. At right, free trophozoites of *S. diploidea* are present in viable brain tissue (arrows) at the periphery of the mass. Formalin-fixed, paraffin embedded. Hematoxylin and eosin stain. Scale bar: 100 μm.

Fig. 3. Two trophozoites in the brain specimen are stained bright blue and lie amid many small round blue host inflammatory cell nuclei. Giemsa stain (left). A trophozoite stained with PAS has a brightly speckled staining pattern (right). Scale bar: 50 μm.

Fig. 1. Preoperative (left panels) and postoperative (right panels) magnetic resonance images of the head. T1-weighted post-Gadolinium image shows a 2-cm lesion in the left temporal-parietal region that is partly cystic with ring enhancement (arrow, top left). T2-weighted image shows a bright area surrounding the lesion representing edema (arrow, bottom left). Postoperative images repeated 10 months later show scarring and no lesion progression (right panels, top and bottom).
Dehydration and paraffin embedding of the brain sample introduced artifacts; therefore the technical conditions of the 2 ultrastructural studies differed. Growing conditions of the 2 studies also differed, as the isolates were fed bacteria, while organisms in the brain had ingested the blood cells of a human host. Thus, some ultrastructural features of isolated *S. diploidea*, such as a hyaloplasm (8), were not resolved clearly in the brain specimen.

*S. diploidea* has been isolated on many world continents. Hartmann and Nägler reported the first isolation in 1908 in Germany; the source of their clones was lizard feces (9). The name they assigned to it (*Ameba diploidea*) was a reference to the characteristic double nucleus. In 1958, Noble reported finding viable trophozoites in elk feces and buffalo feces in Wyoming (10). Goodfellow et al collected their soil samples in Cambridge, England in the early 1970s from the edge of a pond “where cattle graze . . . and bovine faeces are often seen.” (8). Thus, the genus is distributed worldwide and the life cycle seems to involve animal feces. About a dozen papers on *S. diploidea* have been published, none of which described pathogenicity in an animal host. The last report pertaining to this organism appeared over 25 years ago on clone 1576/1, which had been isolated in Cambridge, England. The clone was discarded in the 1980s and no repository in the world maintains an active clone.

We suggest that the environment around the subject’s house, a small farm in Texas with a few grazing farm animals, most likely harbored *S. diploidea*. The patient stated that he had handled animals in that environment. His history of a resolving sinus infection prior to exhibiting signs of encephalitis is intriguing because it suggests possible inhalation of organisms through the nasopharynx, leading to sinusitis and spreading to the central nervous system (CNS). A route of entry into the CNS through the nasopharynx would be similar but not identical to the pathway for infection with *Naegleria fowleri*, which enters the brain through the olfactory nerves. The location of this lesion in the patient’s posterior temporal lobe, some distance from the basal frontal lobes, does not support that theory and instead suggests a possible hematogenous pathway. Although the patient worked as a

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**TABLE**

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<thead>
<tr>
<th>Antibody</th>
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<td>Acanthamoeba castellanii</td>
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<tr>
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<td>A. polyphagia</td>
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<tr>
<td>Balamuthia mandrillaris</td>
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<tr>
<td>Entamoeba histolytica</td>
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* Immunofluorescence microscopy was performed, as previously described (5), by Dr. Govinda S. Visvesvara at the Centers for Disease Control and Prevention (Atlanta, Georgia).
Giemsa staining often highlighted the double nucleus of *S. diploidea* trophozoites in the brain (top). Double nuclei contained dark, round nucleoli nested within an ovoid, dark outer membrane (solid arrow). The organism has ingested a host leukocyte (open arrow). A drawing made in 1958 of cultivated *S. diploidea* contains a double nucleus with the same appearance (bottom). A characteristic cytoplasmic vacuole also is illustrated in the drawing. Bottom panel is from *J Protozoology* (ref. 10), reproduced with permission. Scale bar: 25 μm.

costguardsman, we think it is unlikely that he acquired this infection in the Gulf Coast marine environment because the organism resides near fresh water and in soils and animal feces.

The dual nucleus of *S. diploidea* relates to the fact that these organisms reproduce sexually, which is a highly novel feature; however, the precise details of the process are not well characterized. The analysis by Goodfellow et al focused primarily on the vegetative state, and thus, the details of reproduction are not available (8). In culture, 2 amebae become closely aligned to each other, fuse, and secrete a cyst wall. Many but not all of the cysts become uninucleate through an unknown mechanism. Thus, the DNA ploidy of all the stages of reproduction is not clear, as it is not known whether each cell brings a haploid nucleus to a diploid zygote. The process of encysting occurred over a period of months in culture. The timing of these life cycle events is clearly relevant to the organism's potential pathogenicity, but we cannot speculate as to whether sexual reproduction occurred in the human host. Many organisms do not display a dual nucleus in culture or in histological sections. It is possible that uninucleate cysts might form in *S. diploidea* infection, but we did not observe them in this case. Cyst forms
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Fig. 7. Transmission electron micrograph shows the highly distinctive short filaments connecting 2 nuclei within a trophozoite in the brain biopsy (top). The micrograph at bottom shows exactly the same feature in isolated S. diploidea. “Where nuclei of a pair are flattened against each other, poorly defined fibrils bridge the gap between their membranes” (8). Technique used in this comparison was not identical. Growth conditions were not the same and there are fixation artifacts in the top panel not present in the comparison panel. Quotation and bottom micrograph are from Protistologica (ref. 8), reproduced with permission. Scale bar: 1 μm.

Fig. 8. Transmission electron micrographs from a trophozoite in the brain specimen (left) and a trophozoite cultivated from nature (right). Both contain mitochondria with a distinctive tubular crystal pattern (m). Dark structures labeled “b” are bacteria that the cultivated organism has ingested. From Protistologica (ref. 8), reproduced with permission. Scale bar: 1 μm.

Fig. 6. A transmission electron micrograph of a trophozoite retrieved from the subject’s brain (top) is compared to a micrograph of S. diploidea that was isolated from nature and placed in culture (bottom). The organism in the brain shows the characteristic double nucleus, a large cytoplasmic vacuole (*), a dense Golgi network (g), and a partly ingested host erythrocyte (rbc). In the bottom panel, S. diploidea isolated from nature also contains a double nucleus (n) and a contractile vacuole (v). The intracytoplasmic round structures with dark rings in the bottom panel are bacteria ingested by the cultivated organism. Technique used in this comparison was not identical. Growth conditions were not the same and there are fixation artifacts in the top panel that are not present in the comparison panel. Bottom panel is from Protistologica (ref. 8), reproduced with permission. Scale bar: 5 μm.
are a known component of GAE due to Acanthamoeba species and are not observed in PAE due to Naegleria fowleri (1, 2).

The discrete nature of the lesion caused by S. diploidea stands in sharp contrast to the more fulminant infection produced by N. fowleri. Even though this lesion was solitary in the brain, we refrained from employing the nomenclature “amebic abscess” as applied to brain lesions caused by Entamoeba histolytica. A classically layered abscess wall was not present and isolated organisms often were present in viable brain tissue at the peripheral margins of the lesion (Fig. 2). The lack of spreading throughout the CNS may simply reflect the earliest stage of lesion development at the time when surgery was performed. Formation of an abscess wall or a more widespread dissemination both could occur later as the lesion matures. Thus, the apparently favorable outcome in this case does not adequately establish the prognosis for future cases.

The essential features to consider when diagnosing and treating S. diploidea infection of the CNS are as follows. 1) It presented as a circumscribed tumor-like mass lesion with no abscess wall in the brain of a previously healthy person. 2) It had central necrosis, hemorrhage, and an inflammatory exudate that contained acute and chronic inflammatory cells, but no eosinophils or granuloma formation. 3) It contained perivascular and diffuse collections of 40- to 70-μm-wide amebae with a distinctive double nucleus, the nucleoli of which were exhibited well in frozen sections stained with hematoxylin and eosin. 4) The lesion contained isolated trophozoites in viable brain parenchyma on the periphery of the mass away from areas of inflammation and necrosis. 5) Trophozoites ingested the blood cells of the host, stained bright blue with Giemsa, and displayed a red speckled pattern with PAS staining. 6) The prognosis after treatment with surgical excision and drugs was favorable in this instance, which differs from other types of amebic infections of the brain. In the future, immunoreagents need to be produced and genomic sequencing undertaken on S. diploidea so that molecular and diagnostic tests can be performed. To that end, in June 2000 the primary author collected soil samples at Coe Fen, at the precise location where clone 1576/1 was collected at Cambridge, England (8). The attempt to isolate S. diploidea from these samples at the CDC was not successful.

ACKNOWLEDGMENTS

We acknowledge the written permission, granted by current copyright custodians, to reproduce the venerable figures from Protistologica (8) and Journal of Protozoology (10). Prescient suggestions of David H. Walker, MD, pertinent to the diagnosis and ideas of Dr. Francine Marciano-Cabral concerning antimicrobial treatment are acknowledged. Preliminary morphological findings were presented at the 76th Annual Meeting of the American Association of Neuropathologists, Inc., Atlanta GA, June 9, 2000. A preliminary announcement of the organism’s pathogenicity has appeared in a letter to the editor in JAMA in 2001 (7).

REFERENCES