Direct Visualization of Fungal Infection in Brains from Patients with Alzheimer's Disease

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Accepted 23 June 2014

Abstract. Recently, we have reported the presence of fungal infections in patients with Alzheimer's disease (AD). Accordingly, fungal proteins and DNA were found in brain samples, demonstrating the existence of infection in the central nervous system. In the present work, we raised antibodies to specific fungal species and performed immunohistochemistry to directly visualize fungal components inside neurons from AD patients. Mice infected with *Candida glabrata* were initially used to assess whether yeast can be internalized in mammalian tissues. Using polyclonal rabbit antibodies against *C. glabrata*, rounded immunopositive cells could be detected in the cytoplasm of cells from liver, spleen, and brain samples in infected, but not uninfected, mice. Immunohistochemical analyses of tissue from the frontal cortex of AD patients revealed the presence of fungal material in a small percentage (~10%) of cells, suggesting the presence of infection. Importantly, this immunopositive material was absent in control samples. Confocal microscopy indicated that this fungal material had an intracellular localization. The specific morphology of this material varied between patients; in some instances, disseminated material was localized to the cytoplasm, whereas small punctate bodies were detected in other patients. Interestingly, fungal material could be revealed using different anti-fungal antibodies, suggesting multiple infections. In summary, fungal infection can only be observed using specific anti-fungal antibodies and only a small percentage of cells contain fungi. Our findings provide an explanation for the hitherto elusive detection of fungi in AD brains, and are consistent with the idea that fungal cells are internalized inside neurons.

Keywords: *Candida*, fungal infection, intracellular infection, neurodegenerative disease

INTRODUCTION

A major challenge in medicine at present is uncovering the precise etiology of a number of neurodegenerative diseases. These types of disorders are of paramount importance from a social and economic viewpoint. Alzheimer's disease (AD) is one of the most threatening neurodegenerative diseases, particularly for the elderly [1]. Improvement in the current treatment of AD very much depends on the knowledge

of its etiology. The most accepted theory for the origin of AD is the “amyloid cascade hypothesis” [2, 3]. Extracellular deposits of amyloid- β peptide (A β) constitute a cytological hallmark in AD brains, and the amyloid cascade hypothesis proposes that the initial symptoms of the disease can be explained by the deposition of A β that is produced by an imbalance between the production and clearance of this peptide. However, this concept does not explain some clinical symptoms of AD [4]. We recently reported the existence of disseminated mycoses in AD patients [5, 6]. Accordingly, several fungal macromolecules including fungal proteins and polysaccharides ((1,3)- β -glucan) could be detected in peripheral blood serum from patients, and fungal proteins were found in brain tissue [5]. The

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species of fungi present in these samples were identified by sequence analysis from PCR-amplified DNA. The conclusion from these studies is that mixed fungal infections may be present in the central nervous system (CNS) of AD patients, providing a possible explanation for the variability in the evolution and severity of clinical symptoms observed. In support of this hypothesis, the A β peptide exhibits potent antimicrobial activity against *Candida albicans* and some bacteria [7], pointing to the possibility that secretion of A β is triggered by microbial infection as part of the innate immune response [8]. Indeed, exposure of cultured neuronal cells to spirochetes, or to bacterial lipopolysaccharide, induces deformation of A β peptide and hyperphosphorylation of tau proteins [9–11]. Notably, the antimicrobial peptide β -defensin-1 is upregulated in brain from AD patients [12]. Furthermore, amyloid can be formed from fungal adhesion proteins, as deposits of amyloid plaques on the surface of fungal cells [13, 14]. These amyloid deposits lead to the development of fibrils containing glucans and other polysaccharides, which in turn may interact with human serum amyloid P component that coats fungal cells and hyphae, participating in the evasion of the immune system [15, 16]. The existence of chitin-like structures in brain from AD patients has been demonstrated [17], together with a significant increase in chitinase (chitotriosidase) activity in cerebrospinal fluid (CSF) [18]. Intriguingly, the presence of fungal infection was detected in some patients several years after diagnosis of AD, and antifungal treatment reversed the clinical symptoms [19, 20]. In the present work, we provide further evidence for CNS infection with fungi. Immunohistochemical analyses reveal that a few cells in brains from AD patients contain cytoplasmic material that immunoreacts with antibodies raised against some yeast cells.

MATERIALS AND METHODS

Brain samples from AD patients

The samples analyzed in this study correspond to tissue sections obtained from brain donors diagnosed of AD. Information on these patients is listed in Supplementary Table 1. Morphological data analyzed in this work were assessed by an observer blinded to the diagnosis. The Ethics Committee of Universidad Autónoma de Madrid approved the study. The transfer of samples was carried out according to national regulations concerning research on human biological samples. For all cases, written informed consent

is available. All ethico-legal documents of the brain bank, including written informed consent, have been approved by an ethics committee external to the bank. The brain bank that supplied the brain tissue samples is Banco de Tejidos CIEN, belonging to Instituto de Salud Carlos III, the Spanish National Health Institute (<http://bt.fundacioncien.es/>). General procedures of the brain bank have been published in: Alzheimer Center Reina Sofia Foundation: fighting the disease and providing overall solutions. Briefly, a rapid neuropathological autopsy was performed shortly after communication of the donor's decease by their proxies. Immediately after brain extraction, two symmetrical brain halves were obtained in fresh through a mid-sagittal cut. The right half was frozen in -50°C isopentane after serial cutting of the brain hemisphere, the cerebellar hemisphere and the brainstem. The left half was fixed in 4% phosphate-buffered formaldehyde during 3 weeks, and thereafter it was cut and sampled. A total 25 tissue blocks were obtained and embedded in paraffin.

*Mouse infection with *C. glabrata**

To analyze the appearance of *C. glabrata* in different mouse tissues, nude mice were employed to facilitate yeast infection of different organs. *C. glabrata* was employed for several reasons: this yeast gives rise to intracellular infection of culture cells [23] and also we have obtained rabbit antibodies against this yeast. Four nude mice were infected by intravenous injection of 5.8×10^6 colony forming units (cfu) of *C. glabrata* in 0.3 ml of phosphate-buffered saline (PBS). Control mice remained uninfected. Mice were sacrificed after 90 days, and spleen, brain, and liver were extracted for analysis. This experiment was repeated twice. The organs were fixed and prepared for immunohistochemical analyses as detailed below.

Fungal growth

Fungi (*C. glabrata*, *C. albicans*, and *C. parapsilosis*) were grown in YEP medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C . *Syncephalastrum racemosum* and *Penicillium notatum* were purchased from Allergon AB (Sweden).

Production of polyclonal antibodies

Rabbit antisera against *C. albicans*, *C. parapsilosis*, *C. glabrata*, *P. notatum*, and *S. racemosum* were obtained by inoculation of 1 or 2 mg of dried fungi

(after autoclaving and lyophilization) in 0.5 ml PBS. Each inoculum had been previously mixed with an equal volume of Freund's adjuvant. Rabbits were inoculated up to four times every three weeks and the antibody titer and specificity of the sera were tested by immunohistochemistry and immunoblotting.

Histological analysis

Paraffin sections (5 μ m) were fixed in 10% buffered formalin for 24 h and then embedded in paraffin following standard protocols. Sections (4 μ m) were stained with hematoxylin-eosin, washed in running tap water for 5 min, dehydrated in a series of ethanol/water, cleared in xylene and mounted in DPX (Merck).

To carry out the immunohistochemical analysis, paraffin was removed and the tissues were rehydrated, boiled for 2 min in citrate buffer, and washed for 10 min in ammonium chloride (50 mM) at room temperature. Subsequently, tissue sections were washed with PBS tween and incubated for 20 min with PBS/BSA 2%. Sections were then incubated overnight at 4°C with the various antibodies at a 1 : 500 dilution, washed with PBS and incubated with the corresponding secondary antibody (all from Invitrogen). As indicated in each experiment, donkey anti-rabbit IgG secondary antibody conjugated with Alexa 647 (Invitrogen) was added at a 1 : 200 dilution. Where indicated, brain sections were incubated with anti-tau antibody at a 1 : 100 dilution (T100, Fisher) followed by incubation with secondary antibody donkey anti-mouse IgG bound to Alexa 594 at a 1 : 500 dilution. In other experiments, sections were incubated with anti-tubulin antibody at a 1 : 1000 dilution (Sigma) followed by incubation with secondary antibody donkey anti-mouse IgG conjugated to Alexa 488 at a dilution 1 : 200. Finally, sections were observed under a Zeiss Axiovert 200 microscope.

Confocal microscopy was carried out using a confocal laser scanning microscope LSM710 coupled to vertical microscopy AxioImager.M2 (Zeiss). The spectral system employed was Quasar + 2 PMTs. Zeiss ZEN 2010 Program.

RESULTS

Immunohistochemical analysis of tissues from mice infected with *C. glabrata*

We have previously shown that yeast cells, such as *C. famata*, can attach and enter cultured human cells [21]. These observations are consistent with findings from other laboratories reporting intracellular infec-

tion of mammalian cells by several fungi [22–25]. Initially, we wished to demonstrate that yeast can also be internalized in different tissues in mice. For this reason, we employed nude mice in order to allow a better infection. Thus, nude mice were injected intravenously with live *C. glabrata*. Three months after infection, mice were sacrificed and tissue sections were obtained from various organs and immunostained with anti-*C. glabrata* antibodies. As control, we analyzed uninfected mice and mouse tissue treated with pre-immune serum. Immunohistochemical analysis revealed the localization of fungal cells in spleen, liver and brain samples, appearing as rounded bodies possibly in the cell cytoplasm (Fig. 1). Importantly, no immune-positive staining was detected in tissues from uninfected mice, demonstrating that the antibodies employed do not cross-react with mouse cellular components. Also, no reactivity was found with pre-immune serum. These results illustrate that yeast cells can be detected in mouse tissues and these rounded bodies have a size of about 1 micron.

In order to determine if these rounded bodies that immunoreact with the anti-*C. glabrata* antibodies are intracellular, confocal microscopy was carried out. Brain sections from both uninfected or *C. glabrata*-infected mice were examined. Figure 2 clearly shows that these yeast rounded bodies are intracellular and localize in the cytoplasm, we propose to refer to them as “intramycosomes”. Therefore, the nude mice system serves to provide an idea about the appearance of intracellular yeast cells in brain tissue.

Immunohistochemistry of AD brain samples with anti-*C. glabrata* antibodies

The main goal of this study was to assess the potential presence of fungal components in brain tissue from AD patients using anti-*C. glabrata* antibodies. It must be considered that fungal antibodies were obtained using whole yeast cells comprising many proteins and also polysaccharides, and thus cross-reactivity may occur with related fungal antigens. However, as demonstrated, they do not recognize any component in mouse tissue. Serial sections of the frontal cortex of a human control or an AD brain (AD 1) were stained with hematoxylin-eosin to visualize the typical presentation of neurons (Fig. 3A). Whereas typical frontal cortex neurons were observed in control tissue, frontal cortex sections from AD brains contained less neurons, with apparent gliosis. Additionally, immunohistochemical analysis using antibodies that immunoreact with hyperphosphorylated tau protein revealed the formation

Fig. 1. Immunohistochemical analysis of mouse tissues using anti-*C. glabrata* antibodies. Immunofluorescence of *C. glabrata* grown in YEP medium using rabbit polyclonal antibodies raised against *C. glabrata* (upper panel). Four nude mice were infected with 5.8×10^6 cfu *C. glabrata* by i.v. injection. 90 days later, mice were sacrificed and liver, spleen, and brain were collected. This experiment has been repeated twice with four animals each time. Uninfected animals served as a control. Sections were obtained from fixed tissue and immunohistochemistry was performed. Sections were incubated with anti-*C. glabrata* antibodies (1 : 500 dilution) or with preimmune serum as indicated in the Figure, followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (1 : 500). Scale bar: 20 μ m. At least, 15 different fields were examined in each sample (400x magnification).

Fig. 2. Confocal microscopy of brain sections from *C. glabrata*-infected nude mice. Four nude mice were infected with 5.8×10^6 cfu *C. glabrata* by i.v. injection. 90 days later, mice were sacrificed and brains were collected. This experiment has been repeated twice with four animals each time. Uninfected animals served as a control. Sections were obtained from fixed tissue and immunohistochemistry was performed. Brains were incubated with anti-*C. glabrata* antibodies (1 : 500 dilution) or with preimmune serum (1 : 500) as indicated in the Figure, followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (1 : 500). Afterwards, sections were incubated with Dapi 1 μ g/ml. The different panels in the figure are indicated. Merge is the result of mixed red and blue panels. Brains were observed under a Confocal laser scanning microscope LSM710 coupled to vertical microscopy Axiolmager.M2 (Zeiss). Scale bar: 30 μ m. At least, 15 different fields were examined in each sample (400x magnification).

of tangles in AD tissue sections, but not in control brain (Fig. 3A). Notably, double immunofluorescence staining of AD brain sections using anti-tubulin and anti-*C. glabrata* antibodies demonstrated the presence

of microtubules in the cytoplasm (green), and also small punctate bodies (red) that immunoreacted with the antifungal antibodies and seemed also to localize in the cytoplasm (Fig. 3B). This morphology was

Fig. 3. Immunohistochemical analysis of control and AD brain sections. A) Hematoxylin-eosin staining of brain sections (frontal cortex) from control patient 1 and AD patient 1 (AD1) observed under a Zeiss Axiovert 200 microscope (200x magnification) (upper panels). Immunohistochemistry of hyperphosphorylated tau protein using a specific anti-tau antibody (1 : 100 dilution), followed by incubation with secondary antibody donkey anti-mouse IgG conjugated to Alexa 594 (1 : 500) (lower panels). B) Immunohistochemistry using anti-tubulin and anti-*C. glabrata* antibodies in control 1 and AD1 brain sections. Sections were incubated with anti-tubulin (1 : 1000 dilution) and anti *C. glabrata* (1 : 500) antibodies, followed by incubation with secondary antibody donkey anti-mouse IgG conjugated to Alexa 488 (green) (1 : 200) and incubation with donkey anti-rabbit IgG conjugated to Alexa 647 (red) (1 : 500 dilution), respectively. The different panels in the figure are indicated. Merge is the result of mixed green and red panels. Scale bar: 20 µm. At least, 15 different fields were examined (630x magnification). C) Immunohistochemical analysis of brain sections from different AD patients. Sections were incubated with anti-*C. glabrata* antibodies (1 : 500 dilution) and anti-tubulin (1 : 1000), followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (red) (1 : 500) and with secondary antibody donkey anti-mouse IgG conjugated to Alexa 488 (green) (1 : 200), respectively. The different panels of the figure from left to right are: phase contrast, tubulin, anti-*C. glabrata* and merge. Merge is the combination of green (tubulin) and red (anti-*C. glabrata*) panels. Scale bar: 20 µm. At least, 15 different fields were examined (630x magnification).

not observed in control brain sections, demonstrating that anti-*C. glabrata* antibodies do not recognize any component in human brain. Importantly, the secondary anti-rabbit antibodies employed were conjugated to Alexa 647, avoiding the autofluorescence of human brain tissue. Significantly, the immunopositive punctate material was probably intracellular and seemed to be present in the cytoplasm of neurons (see below). Indeed, the morphology observed was reminiscent of the staining produced after infection of mice with *C. glabrata* (Fig. 1). Crucially, the immunopositive material was not present in all neurons; approximately 10% of neurons exhibited this punctate morphology. Of note, the existence of this intracellular material was only apparent using specific antibodies, and may explain why it has not previously been reported, although some similarities with chitin staining are clear [17].

The possibility that fungal material co-localizes with amyloid plaques remains to be investigated. However, it should be taken into consideration that these plaques represent a host response and the secreted peptides may accumulate in regions distant to the infection sites.

Analysis of different AD patients employing anti-C. glabrata antibodies

We next extended this analysis and tested frontal cortex tissue from an additional three patients (AD 2–4) using double immunofluorescence staining to visualize tubulin and fungal material. The results presented in Fig. 3C clearly demonstrate that immunopositive material could be observed in other AD patients. Moreover, the particular morphology found with anti-*C. glabrata* antibodies varied from patient to patient. Thus, intracellular bodies of about 1 micron were evident, but the number and intracellular distribution of these immunoreactive bodies differed. In one patient (AD 4), a typical punctate cytoplasmic material chiefly located surrounding the nucleus was observed, while staining distributed in the cytoplasm was visualized in a second patient (AD 2). In another patient (AD 3), the number of cytoplasmic bodies was lower. In summary, while the morphology observed with the antifungal antibodies differs between patients, in all samples tested, only a small percentage of cells are positive for the presence of these fungal-related components. Importantly, no immunopositive bodies were found in control samples, indicating that the anti-*C. glabrata* antibodies did not recognize neuron components.

In order to assess if this fungal-related material observed in brain tissue from AD patients was intra-

cellular, confocal microscopy was carried out. Figure 4 shows the intracellular bodies and the cytoplasmic distribution in patient AD 1. In conclusion, the fungal-related material resembles that observed in brain mice and it is clearly intracellular. This may be another reason to account for the elusiveness to detect this infection.

Comparative immunohistochemical analysis using different antifungal antibodies

Our previous work suggested that AD patients may present different fungal infection, and also that mixed fungal infections can occur in the same patient [5]. We therefore assessed whether frontal cortex tissue from AD brains could immunoreact with antibodies raised against other fungal species. To this end, we obtained rabbit polyclonal antibodies against *Penicillium notatum*, *Syncephalastrum racemosum*, *C. albicans*, and *C. parapsilosis*. Clearly, these antibodies can recognize different antigens, but it is also possible that the recognition of some fungal antigens may be common to all fungi. As before, no immunoreactivity was observed in control brain sections (not shown), indicating that potential antigens for the rabbit polyclonal antibodies were absent in neural cells. Interestingly, the anti-*P. notatum* antibody detected small intracellular bodies localized most probably to the cytoplasm surrounding the nucleus in a few neurons (Fig. 5). This punctate morphology did not appear in neighboring neurons, further supporting the notion that only a few cells are infected. Additionally, punctate bodies immunostained with the anti-*P. notatum* antibody were detected in a blood vessel (Fig. 5), suggesting that capillaries may also be infected by fungi. Indeed, it is well established that fungi can directly infect blood vessels, provoking vasculitis in the CNS [26, 27]. Neurovascular inflammation present in the vast majority of AD patients [28, 29] could conceivably be explained by direct fungal infection of blood vessels. Staining with antibodies to *S. racemosum* and *C. albicans* also revealed a number of punctate bodies around and inside neurons (Fig. 5). Again, many neurons were immunonegative, and thus served as an internal control for the specificity of the antibody. As indicated, it is plausible that the different antibody preparations employed immunoreact with different fungal cells or components. If mixed fungal infection exists in a single patient, perhaps the different antibodies detect distinct infected neurons. Alternatively, if there is cross reactivity between these antibodies they could detect common antigens. Nevertheless, the

Fig. 4. Confocal microscopy of brain sections from control and AD1 patient. Brain sections (frontal cortex) from control 2 and AD patient 1 (AD1) observed under a Confocal laser scanning microscope LSM710 coupled to vertical microscopy AxioImager.M2 (Zeiss). Sections were obtained from fixed tissue and immunohistochemistry was performed as described in the legend of Fig. 3. Sections were incubated with anti-*C. glabrata* antibodies (1 : 500 dilution) or with preimmune serum (1 : 500) as indicated in the Figure, followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (1 : 500). Afterwards, sections were incubated with Dapi 1 μ g/ml. The different panels in the figure are indicated. Merge is the result of mixed red and blue panels. Scale bar: 30 μ m. At least, 15 different fields were examined in each sample (400x magnification).

use of different anti-fungal antibodies serves to highlight the presence of punctate material that exists in some neurons. Thus, these data reinforce the notion that the observed bodies are of fungal origin. Of note, an antibody raised against *C. parapsilosis* failed

to immunoreact with AD samples, and the pattern observed was similar to that of control samples. This negative result is consistent with the idea that the antibody preparations employed recognize specific fungal antigens.

Fig. 5. Immunohistochemical analysis of brain sections from AD patient 2 (AD2) using rabbit polyclonal antibodies raised against different fungi (*Pnotatum*, *C.albicans*, *S.racemosum*, and *C.parapsilopsis*). Sections were incubated with anti-fungal antibodies (1 : 500), followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (1 : 500 dilution). The different panels of the figure from left to right are: Phase contrast and red (anti-fungal antibody). Scale bar: 20 μ m. At least, 15 different fields were examined (630x magnification).

DISCUSSION

The possibility that AD is caused by microbial infection has been proposed by a number of investigators. Both viruses and bacteria have been alleged to be the causal agents of this disease [30, 31], including herpes viruses [32]. Also, several bacteria, including *Chlamydophila pneumonia* and spirochetes, have been detected in AD patients [33–35]. Recently, we have suggested that fungal infections may represent a risk factor, or possibly the cause of AD [5, 6]. We provided evidence that fungal macromolecules are present in peripheral blood from the vast majority of AD patients examined. In addition, fungal proteins were identified in CNS tissue from AD patients after polyacrylamide gel electrophoresis and mass spectrometry analysis, and fungal DNA was detected by PCR. These observations are consistent with disseminated fungal infections affecting AD patients. Our present results using specific antibodies to detect fungal material inside neurons add further support to the concept that AD patients are infected with fungi. Consistent with this idea, chitin-like material has been detected in brain tissue from AD patients [17]. It is conceivable that the calcofluor staining method employed to detect chitin identified intracellular fungal cells, although this possibility was not considered. It is noteworthy that antifungal treatment reversed the clinical symptoms of some patients diagnosed with AD [19, 20]. Additionally, the cytokine and inflammatory molecule profile found in AD patients is reminiscent to that elicited by fungal infection [36, 37].

Several possibilities could explain the detection of fungal macromolecules in AD brains. One possibility is that, for reasons yet to be determined, AD patients are prone to fungal infection. This may ostensibly be due to various reasons, e.g., a dysfunction of the immune system, genetic background, age, and way of life, etc. Another possibility is that contamination can occur when obtaining and processing brain tissue. However, no fungal macromolecules were found in control brain, supporting the view that these operations do not necessarily lead to fungal contamination. Moreover, the detection of fungal proteins by proteomic analyses is difficult to explain only if contamination exists. Nevertheless, our finding that only a small percentage of neurons immunoreact with antibodies raised against fungi rule out the possibility that this is due to contamination, particularly if it is considered that the immunoreactive material is located inside neurons. It can also be thought that the fungal-related material described in this work corresponds to fungal remnants

of a fungal infection that occurred before AD and that this material is unrelated to the pathology of the disease. However, fungal material diminishes in culture cells infected with yeast a few days after entry [21]. Finally, the most likely possibility in our opinion could be that AD patients are indeed infected by fungi, which cause this chronic and progressive disease. To the best of our knowledge, there are no reasons to discard this idea. Moreover, a number of well-established observations in AD are consistent with the hypothesis that fungi are the etiological agents of AD. As indicated previously, AD is a chronic and progressive disease, as occurs in fungal infection if untreated. Also, the severity and evolution of clinical symptoms can vary widely between different patients, which could be explained by different fungal species or different combinations of mixed fungal infections. Additionally, the vascular pathology observed in AD patients could be caused by direct infection of blood vessels by fungi. Strikingly, immunoreactive material can also be observed around blood vessels, which could explain the vascular modifications observed in the majority of AD patients. Therefore, it is plausible that the chitin-like material described previously in close connection to blood vessels in AD [17] is due to fungal infection. It is well accepted that neuroinflammation occurs in AD, with infiltrates of T-lymphocytes and monocytes that are responsible, together with microglia cells, for the increase in a number of cytokines in the CNS [38–40]. Systemic inflammation also exists in AD patients, with the production of a number of interleukins and other cytokines that are increased in peripheral blood many years before the initiation of cognitive decline [41, 42]. In this context, increased levels of IL-1 α , IFN- γ , TNF- α and reactive C protein have been described in fungal infections and in AD peripheral blood and CNS after microglia activation [36, 37, 40, 43–45]. Notably, IL-17A, which plays a pivotal role in the immune response against fungal infections [46–48], is also increased in AD T-lymphocytes [49, 50]. This increase in the immune response supports the possibility that chronic fungal infections are present in AD patients [37]. The genetic susceptibility of individuals to different fungal infections is also in accord with the existence of familial susceptibility to AD in a small percentage of the population [51].

The fact that yeast can enter mammalian cells giving rise to a rounded morphology, together with their small size (which can be 1 micron or less), makes the detection of fungi challenging. Also, the existence of these intracellular forms of fungal cells cannot be easily demonstrated unless specific reagents are employed.

This difficulty is greater if only a small percentage of neurons and other brain cells are infected. Collectively, these concepts may provide an explanation for the hitherto elusive detection of fungal infections in neurodegenerative diseases, such as multiple sclerosis or AD. There is a paucity of studies on intracellular infection of non-phagocytic cells by fungi [21]. The majority of these studies have been carried out with monocyte cell lines and macrophages [22–25]. Future studies in this direction will benefit from the development of new antibodies and other reagents that will serve to examine the presence of fungal infections in neurodegenerative diseases.

ACKNOWLEDGMENTS

The financial support of Fundación ONCE (Organización Nacional de Ciegos Españoles) is acknowledged. We acknowledge an institutional grant to Centro de Biología Molecular “Severo Ochoa” from the **Fundación Ramón Areces**.

Authors' disclosures available online (<http://www.j-alz.com/disclosures/view.php?id=2405>).

SUPPLEMENTARY MATERIAL

The supplementary table is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-141386>.

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