Fungal Infection in Patients with Multiple Sclerosis

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Abstract: Multiple sclerosis is a chronic demyelinating disease of the nervous system that may provoke a variety of symptoms, including motor and sensory dysfunctions. The infectious nature of this devastating disease has been proposed. We have investigated for the presence of fungal infection in patients diagnosed with multiple sclerosis. Our results provide evidence that in all seven patients studied there are signs of fungal infection. Thus, some of these patients exhibit high antibody titers against several Candida spp. In addition, quantitative PCR indicates that six out of seven patients contain fungal DNA in blood. Four contain significant amounts of β-1,3 glucan in serum, while the presence of fungal antigens was evident in practically all of them, though to different extents. Yeast antibody and antigen analyses reveal the presence of both in cerebrospinal fluid. Two possibilities can be put forward to account for these findings: one is that fungal infection causes multiple sclerosis; another is that fungal proliferation occurs as a result of immune system dysfunction.

Keywords: Multiple sclerosis, fungal infection, candida antibodies, β-1,3 glucan, candida PCR.

INTRODUCTION

Multiple sclerosis is a demyelinating disease of the central nervous system (CNS) leading to the formation of sclerotic plaques in the brain and spinal cord [1-3]. This complex disease is thought to be triggered by an interaction between genetic and environmental factors [2, 4-6]. Genetic susceptibility to MS is determined by the HLA-DRB1*1501 class II allele and its interactions with other HLA class II alleles, while other genes play a minor role [4, 7]. MS is prototypical of inflammatory CNS diseases and provokes a variety of clinical symptoms. Motor impairment and sensory organs dysfunction are two major problems associated with MS. Other symptoms include spasticity, fatigue and bladder dysfunction [8-12]. Spasticity in patients with upper motor neuron lesions is due to an abnormal integration of the nervous system motor responses to sensory input, leading to the development of fixed muscle contractures [11]. Fatigue, a common symptom in MS patients, may be caused by diffuse axonal damage and brain atrophy [9, 12]. Bladder dysfunction, another common symptom, may result from spinal cord damage and has a negative impact on life quality [10]. Typically, this chronic disease is recurrent, presenting relapsing-remitting episodes. MS is more prevalent in northern Europe and affects twice as many women as men. It is estimated that about 5 million people are affected worldwide [8]. The exact cause of MS has been the object of intensive research in many laboratories, although the etiology of MS remains enigmatic [13, 14]. Autoimmunity has been put forward as a plausible cause of this devastating disease [15, 16]. Research in animal models indicates that the presence of autoreactive T cells can result in inflammatory demyelination of the CNS, although similar numbers of myelin-reactive T cells are found in MS patients and healthy subjects [3, 13]. Autoimmunity may result from the presence of viral proteins or other pathogen antigens that mimic self-protein molecules in the CNS [15-17]. These antigens can prime genetically susceptible individuals, leading to an immunologic challenge and disease through bystander activation by cytokines. However, no association between antimyelin antibodies and progression to multiple sclerosis exists [18]. Moreover, a disorder of the immune system that attacks oligodendrocytes does not account for some clinical observations [15]. For instance, the existence of distinct foci of degeneration cannot be explained by indiscriminate aggression against glial cells [3, 19]. Furthermore, blood vessel inflammation is not easily explained by autoimmunity and destruction of nerve cells. Intensive research to find an infectious agent that directly provokes or triggers MS has been carried out in many laboratories [13, 14, 16, 20, 21]. A number of viruses, mostly from the herpesvirus group, have been proposed as a possible culprit [22-26]. Some investigators have suggested that MS may be caused by bacteria such as *Chlamydia pneumoniae* [17, 20].

We have suggested that *Candida famata* is the etiologic agent of acute zonal occult outer retinopathy (AZOOR) [27-29]. This ocular disease was thought to be an autoimmune disorder that affects the optic nerve and the retina [30, 31]. Some AZOOR patients may also present clinical symptoms in the CNS [31]. Our previous results, prompted us to investigate the existence of fungal infection in MS patients. Our findings provide evidence for the presence of fungal infection in these patients.

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MATERIALS AND METHODS

Participants

Seven MS patients were selected to analyze the presence of fungal infection. The incapacity scale (EDSS) for these patients was: 7.0 (patients 1, 3, 4 and 5); 6.0 (patient 7), 5.0 (patient 2) and 2.0 (patient 6). All these patients were informed about the use of their samples, and their written consent was obtained. Ten healthy controls were also analyzed. The research was carried out in accordance with the Helsinki Declaration.

Yeast Growth

Yeasts were grown in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) by incubation at 30°C. The same medium, containing agar, was used to isolate individual yeast colonies.

Antibodies

Rabbit antisera against different yeast species were obtained by inoculation of 0.5 ml of phosphate buffered sorbitol (PBS) containing 1 or 2 mg of yeast after autoclaving and lyophilization. Each inoculum had been previously mixed with the same volume of Freund's adjuvant. Swiss albino rabbits were inoculated up to four times with a three- four weeks interval and the antibody titer and specificity of the sera were tested by immunofluorescence and Western blotting.

Immunofluorescence Assay

For C. famata, 1 ml of culture was placed in 1.5 ml microcentrifuge tubes. Cells were washed with PBS, incubated with 50 mM ammonium chloride for 10 min, and washed three times with PBS-Tween 20. Cells were then treated with the different sera diluted 1:500 and 1:5, respectively, in PBS-Tween 20, at 37ºC for 2 hours, washed again with PBS-Tween 20 and incubated with the secondary antibody. Rabbit anti-human immunoglobulins IgG+IgA+IgM (Sigma) fluorescein-conjugated antibody was added at a 1:500 dilution. The cells were then washed, resuspended in PBS and mounted on slides with a drop of Depex (Serva). Finally, the cells were observed under a fluorescence microscope. For the remaining Candida species, *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*, the commercial kit Euroimmun (Medizinische Labordiagnostika AG) was used in accordance with the manufacturer's instructions and using the same serum dilutions as for *C. famata*.

DNA Extraction from Whole-Blood Specimens

The DNA was extracted from blood according to the following method. One ml of blood was centrifuged at 20000g for 20 minutes. Pellets were resuspended in 1 ml of tri-distilled filtered water and incubated at room temperature for 20 minutes. Samples were centrifuged for 3 minutes at 20000g and washed twice more with tri-distilled filtered water. Pellets recovered from the last centrifugation were resuspended in 300 µl of PBS. Samples were boiled for 10 minutes and then incubated for 2 hours at 37ºC with 15 µl of Zimolase (ICN) (20mg/ml) and for a further 2 hours at 58ºC with 30 µl proteinase K (Sigma) (10mg/ml). Then, 200 µl of a detergent buffer [Tris-HCl 10 mM (pH 7.4), Cl2Mg 1 mM, NP40 al 0,5% y Tween20 al 0,5%] were added and samples were boiled again for 10 minutes before adding 1 ml of phenol:chloroform (1:1) (Amersham) and centrifuged at 20000g for 20 minutes. The upper aqueous phase was recovered and washed twice with ethyl ether. The DNA was precipitated by addition of 3 volumes of absolute ethanol (Merck) (−20°C) to the aqueous phase. After storing the samples at −20°C overnight, the DNA was centrifuged at 20000g for 20 min. Pellets were dried and resuspended in H2O. DNA from extracts was quantified by The NanoDrop® ND-1000 UV-Vis Spectrophotometer.

Negative controls included three tubes of tri-distilled filtered water and one tube of whole blood from a healthy volunteer for every four patient specimens.

Sequence Analysis for the Design of the Primers and Probe

Sequences of 18s and 5.8s rRNA genes and internal transcribed spacer 1 (ITS 1) region from several organisms (*D. hansenii, P. guilliermondii, C. albicans, C. parapsilosis, C. glabrata, R. mucilaginosa, C. neoformans* an *H. sapiens*) were accessed *via* the GenBank database and were aligned by using the Sequence Analysis Package (Clustal W). Multiple potential primer-binding sites for the panfungal primer pair were chosen by comparing regions of Candida homologous with regions of the fungal group from the fungal kingdom with the most divergent DNA sequences and regions of Candida incongruous with the human DNA sequence. The primer selection was optimized for melting temperature equivalence, lack of duplex, hairpin, or primer dimer formation, and internal stability by using OLIGO software (Amplify) Buscar referencias.

PCR Analyses

The measures used to avoid PCR assay contamination included the use of separate rooms and glassware supplies for PCR setup and products, aliquoted reagents, positive-displacement pipettes, aerosol-resistant tips and multiple negative controls.

Real time quantitative PCR was carried out in an ABI PRISM 7000 thermocycler (Applied biosystems). The reaction mix was prepared with 0.9 µM of each oligonucleotide (forward primer [TGAACCTGCGGAAGGATCAT] and reverse primer [ACGCAGCGAAATGCGATA]) and 0.25 µM of TaqMan probe [G-FAM-TCAACAACGGATCTCTT GG-MGB] in a final volume of 20 µl, to which 50 ng of DNA were added. The concentration of DNA template was normalized by prior PCR with the quoted oligonucleotides, in which DNA was denatured at 95ºC for 10 minutes and amplified in 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Data were analyzed using the SDS 7000 (1.1) software. The *in vitro* sensitivity was 100-1000 RNA copies/ reaction.

Slot-Blot Analyses

200 µl of different serum or cerebrospinal fluid (CSF) dilutions in TBS were added to each well. Samples were blotted onto a 45 mm nitrocellulose membrane (Bio-Rad), previously hydrated in TBS for 10 minutes using the Bio-Dot SF apparatus (Bio-Rad). After blotting, the membrane was processed and developed as described for Western blotting. The primary antibodies, rabbit polyclonal antibodies

raised against C. famata, C. albicans, C. glabrata, C. parapsilosis, Rhodotorula mucilaginosa or Saccharomyces cerevisiae as described above, were used at a 1:1000 dilution. Secondary antibody, a donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham Biosciences), was used at a 1:5000 dilution.

Detection of Fungal Polysaccharides

The presence of β-1,3 glucan in serum was detected using the Fungitell kit in the Fontlab laboratories (Barcelona, Spain) [32].

ANALYSES TO DETECT FUNGAL INFECTION

Given the lack of a single definitive test to assess fungal infection, different assays were needed. In order to investigate fungal infection in patients diagnosed with MS, we carried out the following analyses in serum or whole blood: 1. Antibodies against different *Candida* species assayed by immunofluorescence. 2. Fungal DNA using quantitative PCR. 3. Fungal polysaccharides (β-1,3 glucans) measured by the commercial Fungitell kit. 4. Presence of *Candida famata* and other yeast antigens estimated by slot-blot analyses.

Table 1. Summary of Patients Analyzed in this Work

Patient	Birth Year	Gender	Comments
MS1	1970	female	wheel chair
MS2	1980	female	some difficulties to walk and talk
MS3	1951	male	wheel chair
MS4	1978	male	wheel chair
MS5	1962	female	slight problems to walk
MS6	1962	female	wheel chair
MS7	1954	female	slight problems to walk

PRESENCE OF ANTIBODIES AGAINST YEASTS

Seven patients diagnosed of MS were selected to analyze the presence of fungal infection. Some patient characteristics are presented in Materials and Methods and Table **1**. To analyze yeast antibodies in serum from MS patients, immunofluorescence assays were carried out against the following *Candida* species: *C. famata*, *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei*. Fig. (**1**) shows the immunofluorescence obtained with control serum from a healthy volunteer as compared to two samples from MS patients. It is evident that the majority of MS patients have a very strong presence of antibodies against yeast (Table **2**). The *Candida* species recognized by these antibodies vary from patient to patient, thus, some patients possess high antibody titers that immunoreact with several yeast species, whereas others have a rather narrow antibody response. None of the sera from the ten healthy donors used as controls immunoreacted with any of the Candida species. The presence of antibodies certainly suggests a disseminated yeast infection or colonisation, although their absence cannot definitively rule out this type of infection [33]. The differences in the antibody response observed between patients may reflect the presence of different yeast infections. Alternatively, these differences may arise from variations in the immune response of each patient to fungal infection. We must stress that the presence of these antibodies is indicative of an ongoing yeast infection but not conclusive.

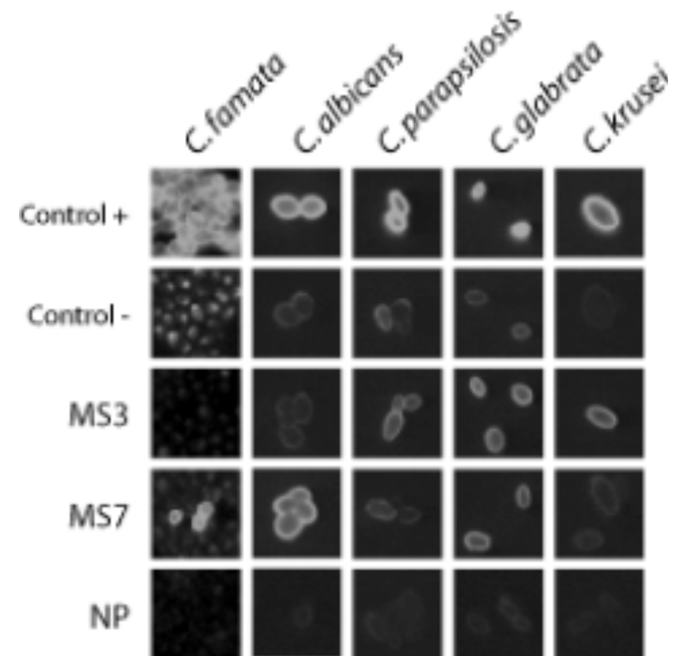

Fig. (1). Immunofluorescence analysis of the presence of antibodies against different yeast species in patients' sera. In the case of *C. famata*, the protocol described in Materials and Methods was followed. As positive control (Control +), rabbit antiserum against *C. famata* was employed; as negative control (Control -), PBS was added instead of primary antibody. For the remaining yeast species, the commercial kit Euroimmun was used. In this case, the controls were those provided by the commercial kit. MS, Multiple sclerosis patient; Control, healthy donor, used as a negative control.

Table 2. Presence of Serum Antibodies Against Different Candida SPP

Patient	CF	CA	CP	CG	CK
MS1	++	++++	-	+++	-
MS2	++	+++++	++++	++++	+
MS3	-	-	+	+	++
MS4	+++++	+++++	+++	+	-
MS5	+	+++++	+++++	+++++	++
MS6	-	-	-	-	-
MS7	++	+++	-	+	-
Controls	-	-	-	-	-

CF: *C. famata*, CA: *C. albicans*, CP: *C. parapsilosis*, CG: *C. glabrata*, CK: *C. krusei*.
Same fluorescence as positive control was marked with +++++, and same as negative control with -.

QUANTITATIVE PCR ASSAYS OF BLOOD

Detection of yeast genomes in peripheral blood provides compelling evidence of disseminated fungal infection. Yeast

cells can be phagocytosed and so lose viability, but the presence of genomes can still be revealed by PCR. Therefore, DNA was extracted from whole blood and quantitative PCR was performed using the appropriate oligonucleotides. All patients, except one (patient 2) had fungal DNA in circulating blood (Table **3**). PCR of DNA extracted from the ten healthy donors used as controls was negative. These findings strongly suggest that disseminated fungal infection is present.

Table 3. Summary of Results of Quantitative PCR and β-Glucan Measurement

Patient	PCR (rRNA Gene Copies/ml Blood)	Fungitell	
(glucan pg/ml)			
MS1	5423	95	+
MS2	NEG	162	+
MS3	1746	62	UNC
MS4	5596	236	+
MS5	4979	48	-
MS6	6708	ND	ND
MS7	2123	312	+
Controls	NEG	37 ± 20.9	-

Glucan levels were considered HIGH for values higher than 80 pg/ml; uncertain (UNC), between 80 and 60 pg/ml; NEGATIVE (NEG) for values smaller than 60 pg/ml; ND: no data. The "controls" value is median value of the ten healthy controls for the beta-glucan assay.

ANALYSIS OF β-1,3 GLUCANS IN SERUM

Another assay that is increasingly being employed to diagnose a fungal infection uses the Fungitell kit. This assay measures β-1,3 glucans, typical fungal polysaccharides that are not synthesized by humans. The results, included in Table **3**, indicate that four of the patients' sera were positive in this assay, while one of them was in the middle range (patient 3) and another was negative (patient 5). Once again this assay points to the possibility of a disseminated fungal infection in at least five patients. It should be remarked that none of these patients was on dialysis, nor had taken any medication that could give account for the positiveness of β-1,3 glucans in serum.

YEAST ANTIGENS ANALYZED BY SLOT-BLOT ASSAY

One of the most sensitive tests to assess the presence of yeast antigens in serum is the use of the slot-blot assay. In this test, different serum concentrations are transferred to a nitrocellulose membrane and immunoblotted using a polyclonal rabbit antiserum raised against *C. famata* or other yeast species. Three patients (1, 5 and 7) were positive when *C. famata* antibodies were employed (Table **4**). This finding indicates that antigens related to this yeast specie were present in peripheral blood when the sample was taken. To further analyze yeast antigens from other species, the slot-blot assay was carried out employing different polyclonal rabbit antibodies. Sera from the ten healthy donors used as negative controls were negative or had low levels of antigens. Notably, large amounts of antigens related to *C. albicans*, *C. parapsilosis*, *C. glabrata* and *R. mucilaginosa* were detected in patient 4, whereas no *S. cerevisiae* antigens were found. In addition, patient 2 had *C. parapsilosis*-related antigens, patient 3 was positive for *R. mucilaginosa* and patient 7 was positive for both *C. albicans* and *C. parapsilosis*. In conclusion, only patient 6 was negative for the presence of antigen against all the antibodies tested.

Table 4. Serum Antigens Immunoreacting with Antibodies Against Different Yeast Species, Analyzed by Dot-Blot

Patient	CF	CA	CP	CG	SC	RM
MS1	52	12	10	7	0	11
MS2	8	11	15	12	1	14
MS3	17	7	4	5	1	21
MS4	18	27	21	3	1	6
MS5	7	6	9	10	0	9
MS6	18	236	147	48	43	136
MS7	20	5	2	0	0	10
Controls	8.6 ± 3.8	5.8 ± 2.6	1.6 ± 2.6	4 ± 2.2	0.6 ± 0.7	4.2 ± 4.4

CA: *C. albicans*, CP: *C. parapsilosis*, CG: *C. glabrata*,
SC: *S. cerevisiae*, RM: *R. mucilaginosa*.
Different dilutions of sera were blotted to a nitrocellulose membrane, which was incubated with rabbit antisera against different yeast species as described in materials and methods. Numbers are the data of optical density obtained by densitometry of the bands. Antigen levels were considered high when the highest value of optical density was 80 or higher; low, when it was between 50 and 15; uncertain, between 15 and 10; and negative if it was smaller than 10.

The results from these different assays are summarized in Table **4**. Taken together, these results provide strong evidence that all seven patients suffered disseminated fungal infection.

PRESENCE OF ANTIBODIES AND FUNGAL ANTIGENS IN CSF

The presence of oligoclonal IgG bands in the CSF is typical of MS patients [19]. Many studies have suggested that these antibodies recognize myelin, although only recently it has become possible to characterize the antibody response on a molecular level. Such characterization strongly suggests that a B-cell response to a specific antigen is occurring in the central nervous system [34]. It was of interest to test for the presence of antibodies that recognize *Candida* species in CSF. We therefore compared the CSF of patient 4 with that of two controls without MS. The amount of IgG in the CSF of patient 4 was 0.107 mg/ml, ten times lower than that estimated in blood (1.17 mg/ml). Fig. (**2**) shows that CSF of patient 4 has antibodies that immunoreact with *C. albicans*, *C. parapsilosis*, *C. glabrata* and, to a lesser extent, with *C. famata*.

Yeast antigens in CSF were analyzed by a slot-blot assay following the protocol described in Materials and Methods. Notably, *C. famata*–related antigens were present in CSF of patient 4, while these antigens were not observed in the CSF control (Fig. **3**). In summary, there is evidence that the CSF

of the MS patient assayed contains both antibodies and antigens related to *Candida* species.

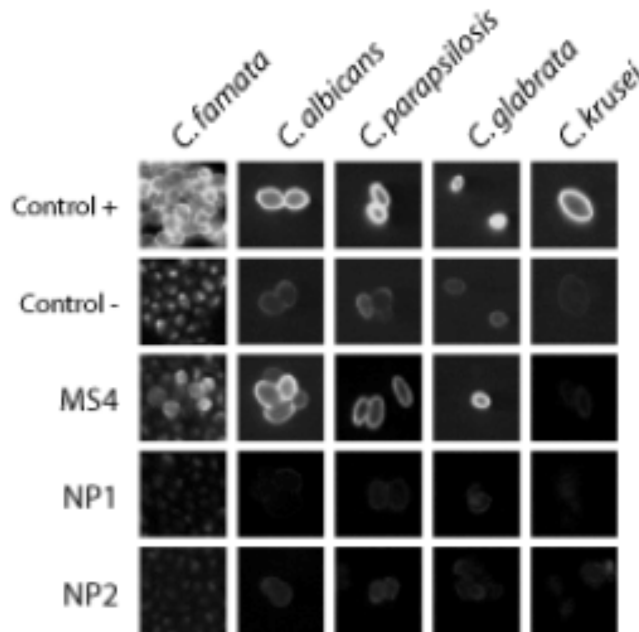

Fig. (2). Analysis by immunofluorescence of the presence of antibodies against different yeast species in CSF from a MS patient. In the case of *C. famata*, the protocol described in Materials and Methods was followed. As positive control, rabbit antiserum against *C. famata* was employed; as negative control, PBS was added instead of primary antibody. For the remaining yeast species, the commercial kit Euroimmun was used. In this case, the controls were those provided by the commercial kit. MS, Multiple sclerosis patient; NP1 and NP2, cerebrospinal fluid (CSF) from healthy donors different from those used for slot-blot and other immunofluorescence assays, used as negative controls.

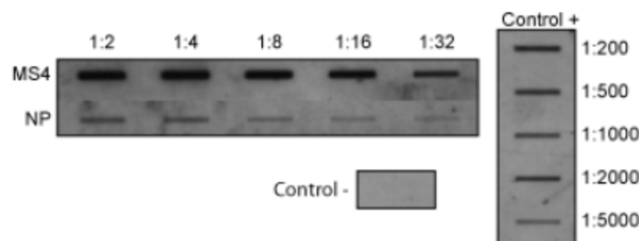

Fig. (3). Presence of *C. famata* antigens in CSF from a MS patient analyzed by slot-blot assay. Different dilutions (indicated in upper part or the right side in the case of control +) of sera were blotted to a nitrocellulose membrane, which was incubated with the rabbit antiserum against *C. famata*. Control +: serum from a patient with chronic candidiasis. Control -: no human serum added. MS, Multiple sclerosis patient; Control by NP.

DISCUSSION

Two possibilities may explain the existence of fungal infection in MS patients. One is that this infection is the actual cause of the disease. Alternatively, infection may be a consequence of immune dysfunction in these patients [13, 14]. Whichever is the case, if fungal infection exists it should be treated appropriately with antifungal compounds. The presence of such an infection, even if it does not cause MS, may negatively affect the clinical course of MS. Patients with MS are usually treated with corticosteroids and other immunosuppressive agents, which may favour fungal infections [35, 36]. In such cases, diagnosis of this type of infection must be taken into consideration in the management of these patients. It is remarkable that all seven patients analyzed in our study showed signs of disseminated fungal infection, detected with non-invasive techniques such as peripheral blood tests.

A number of observations do not permit us to rule out the possibility that fungal infection itself causes MS. Mycoses are usually persistent and recurrent if they remain untreated. A variety of fungi, including species of the genus *Candida*, can infect the CNS provoking demyelination [3, 37, 38]. Routine tests in hospital laboratories to detect yeast infections have a very low sensitivity [39]. Experimental animals infected with *C. famata* survive infection, unlike *C. albicans* [40]. Typically, granulomas develop in some mouse tissues after infection with *C. famata*, even though yeast cells are difficult to detect [40]. These granulomas consist of infiltrates of different immune cells, such as monocytes and neutrophils, which resemble those described in MS [41-43]. Furthermore, granuloma formation has been described in humans and animals infected with different fungal species [41, 43-46]. Another point of interest is that yeast infections are localized in discrete foci and patches, which could explain why the damage to the CNS in MS is restricted to discrete areas [3, 42, 47]. An autoimmune response to a given CNS antigen would result in indiscriminate damage to all tissues bearing this antigen, but this is not observed in MS. In addition, the vascular inflammation described in some MS patients may be the result of yeast colonization, since the tropic characteristics of yeast are conducive to endovascular infection [48, 49]. On the other hand, calcification and tissue sclerosis have been linked with *Candida* growth and infection [50-52]. Thus, it is possible that the formation of plaques in MS may be due to fungal infection. During relapse, the immune response observed in MS patients produces increases in certain interleukins such as TNF, IFN-γ and IL-1, which are indicative of predominance of the Th1 pathway [1, 15, 34, 42]. However, MS patients show a low level of these interleukins during remission. Interestingly, fungal infections can elicit the Th1 pathway with production of TNF, IFN-γ, IL-1, IL-6 and IL-12, leading to protective immunity, or they can induce the production of IL-4 and IL-10 typical of Th2 response, which is associated with disease exacerbation and pathology [15, 53]. Which of these pathways is induced depends on a variety of variables, such as the immune status, the fungal species and the route of infection, among others [54]. The balance between Th1 and Th2 cytokines may be important for optimal antifungal protection [55]. If this equilibrium is destabilized following a Th2 response, the yeast could move from the intestine to the blood to produce an infection. The imbalance in Th1/Th2 could correspond to the relapses and remissions in MS [15, 53, 55].

Another point to consider is that when yeast infections become established at particular locations, tissue sclerosis and low pH may arise due to fungal metabolism, provoking the precipitation of calcium carbonate [51]. This reaction would account for the formation of sclerotic plaques in MS. A genetic predisposition to MS has been established [2, 56, 57]. This is not inconsistent with the possible fungal origin of MS, since the genetic background of a given person may

determine susceptibility of fungal colonization [54, 58]. In summary, to our knowledge, none of the clinical symptoms and observations described for MS patients rule out the possibility that the disease may be caused by a fungal infection. This reasoning, together with the findings reported in the present study, add support to the notion that the etiology of MS may be of fungal origin.

We have reported that the cause of AZOOR was a *C. famata* infection [27]. It was previously thought that AZOOR was also an immune disorder. We have analyzed in detail the evolution of an AZOOR patient over a ten-year period [28]. Despite the administration of different antifungal treatments, total eradication of this disease has not been yet achieved, although a clear diminution of the disseminated candidiasis has occurred. Curiously, a patient suffering from AZOOR and MS has recently been reported [59]. Although some AZOOR patients may have a CNS infection that does not produce the clinical symptoms of MS, it is possible that the genetic makeup of this AZOOR patient predisposes her to the development of MS.

The lack of a universal assay to assess disseminated candidiasis has prompted us to employ a number of techniques to test for this type of infection in MS patients. Thus, high antibody titer against *Candida* species is indicative of an ongoing infection. Treatment of a *C. famata*-infected patient with antifungal compounds led to the disappearance of these antibodies within a few months. The presence of high antibody titers in patients 1, 4, 6 and 7 is indicative of disseminated candidiasis or colonisation. The contrary is not true because antibodies against *Candida* may be absent even during infection depending on the tissues infected, the immune status of the patient, and a series of other factors [60]. In conclusion, to assess candidiasis, the antibody response should be estimated, but in some instances it may not be indicative of infection. More conclusive is the analysis of fungal components in peripheral blood. Notably, in six out of seven patients, fungal sequences were detected in blood stream with quantitative PCR. On its own, this is compelling evidence for disseminated fungal infection. Other assays can also complement the data obtained by PCR. Thus, high levels of β-1,3 glucans were found in three of the patients. Moreover, analysis of circulating yeast antigens suggests that four such antigens related to different *Candida* species were present in four patients. It must be remarked that no *S. cerevisiae* antigens were detected. We interpret this result as an indication that some yeast proteins are secreted into blood stream, allowing detection by a slot-blot assay. Secretion of such proteins may depend on many factors, such as the site of infection, the diet, and the different treatments followed [61]. With all our results taken together, we conclude that there is evidence for disseminated fungal infection in all the seven MS patients analyzed in this work. Remarkably, none of these patients exhibited signs of mucocutaneous candidiasis. Therefore, infection of internal tissues should explain the presence of fungal components in blood stream. Of interest is the finding that the CSF of patient 4 exhibits antibodies that immunoreact with different yeast species. Moreover, the presence of yeast antigens in CSF suggests fungal infection in the CNS. To determine whether mycoses are the cause or a consequence of MS, clinical trials with antifungal compounds must be carried out, although selection of the antifungal compound must be done with care. Before choosing the antifungal compound, the inhibitory efficacy, bioavailability and distribution of the antifungal agent employed in the CNS would need to be considered [62]. Ultimately, such clinical trials could help to determine whether the etiology of MS is of fungal origin, and if this is the case, MS patients may immediately benefit from the use of available antifungal compounds.

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