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Fungal Infection in Patients with Serpiginous Choroiditis or Acute Zonal Occult Outer Retinopathy[▽]

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The etiologies of a number of retinopathies, including serpiginous choroiditis and acute zonal occult outer retinopathy (AZOOR), remain uncertain. Recently, we provided evidence that AZOOR is caused by *Candida famata* infection. The purpose of this article was to investigate the presence of fungal infection in five patients affected with serpiginous choroiditis and five patients with diagnosis of AZOOR. To assess the presence of fungal infection the presence of antibodies in human serum samples against *C. famata*, *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei* was analyzed. In addition, quantitative PCR was carried out to detect fungal genomes in whole blood. Finally, the presence of fungal antigens in the serum samples of patients was investigated. Three AZOOR patients presented high antibody titers against *Candida* spp., while antibodies against *Candida* spp. were observed in serum samples from four patients with serpiginous choroiditis. Fungal genomes in peripheral blood were evidenced in serum samples from one AZOOR and four serpiginous choroiditis patients. Fungal antigens were also apparent in the serum of different patients. Our findings indicate that there was evidence of disseminated fungal infection in most patients examined.

Acute zonal occult outer retinopathy (AZOOR) is an ocular disease characterized by a rapid loss of visual function (13). Typical characteristics of this disease include (i) acute loss of retinal peripheral vision in one or both eyes, (ii) normal funduscopic examination results in the early stages of the disease, and (iii) extensive abnormalities in retinograms whereas evoked potentials are normal (14, 17, 23, 36). A mixed dysfunction of rods and cones is observed, although cones are usually more affected. In the early stages of the disease, visual acuity and angiographic test results are normal despite marked deterioration of the visual field. Some patients exhibit abnormal pupillary reflex, suggesting some type of neuropathy. In fact, inflammation of the central nervous system has been found in a patient with AZOOR (16). In some cases, photophobia occurs, and the appearance of photopsias, referred to as "wavy lights," is common. A typical AZOOR patient is a young myopic woman who is otherwise healthy; indeed, according to a recent study, about 80% of patients with this disease are women (13). Ever since this disease was first described, it has been associated with multiple evanescent white dot syndrome (12, 19, 34, 38). Moreover, similarities between AZOOR, multiple evanescent white dot syndrome, multifocal choroiditis, punctate inner choroidopathy, acute macular retinopathy, and acute idiopathic blind spot enlargement have been described previously (4, 5, 20, 37, 40). Some of these conditions have been associated with histoplasmosis (8, 14). In line with these observations, we reported that AZOOR may be caused by a fungal infection (6). AZOOR was previously considered an immune disorder or a disease caused by an unidentified infectious agent (2, 12, 18).

Serpiginous choroiditis (SC) is a progressive and usually recurrent inflammatory disorder of the choroid, retinal blood vessels, and pigment epithelium. This disease is chronic and usually affects both eyes, leading to vision loss (24, 38). The cause of SC remains unknown, although the possibility that it is an autoimmune disorder has been proposed. In fact, patients suffering from SC are treated with immunosuppressive agents (28, 35). In the present report we provide further evidence that AZOOR and SC may have a fungal origin.

MATERIALS AND METHODS

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

Antibodies. Rabbit antisera against *Candida famata*, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *S. cerevisiae*, and *Rhodotorula mucilaginosa* were obtained by inoculation of 0.5 ml of phosphate-buffered saline (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. Rabbits were inoculated up to four times, and the antibody titer and specificity of the serum samples were tested by immunofluorescence and Western blotting.

Immunofluorescence. 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 serum samples diluted 1:500 in PBS-Tween 20 at 37°C for 2 h, washed again with PBS-Tween 20, and incubated with the secondary antibody. Rabbit anti-human fluorescein-conjugated antibody (immunoglobulin A [IgA] plus IgM plus IgG) was added at a dilution of 1:500. 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, a Euroimmun commercial kit (Medizinische Labordiagnostika AG) was used in accordance with the manufacturer's instructions and using the same serum dilutions as for *C. famata*.

PCR analyses. The DNA was extracted from blood as follows. One milliliter of blood was centrifuged at 20,000 × *g* for 20 min. Pellets were resuspended in 1 ml of triple-distilled filtered water and were incubated at room temperature for 20

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TABLE 1. Summary of patients analyzed in this work

Disease and patient	Age (yr)	Gender	Visual acuity		Present symptom(s)
			Right eye	Left eye	
AZOOR					
AZ1	56	Female	0.4	0.65	Paracentral scotoma; photopsias
AZ2	37	Female	0.95	0.4	Blurred vision; photopsias
AZ3	13	Female	0.1	0.35	Unspecified altered vision
AZ4	58	Male	0.95	0.9	Central scotoma
AZ5	64	Female	0.035	0.15	Blurred vision
SC					
SC1	61	Male	0.09	0.6	Paracentral scotoma
SC2	72	Male	0.01	0.008	Central scotoma
SC3	72	Male	0	0	Central scotoma
SC4	63	Male	0.7	0.05	Central scotoma
SC5	66	Male	0.2	0.9	Central scotoma

min. Samples were centrifuged for 3 min at 20,000 × *g* and washed twice more with triple-distilled filtered water. Pellets recovered from the last centrifugation were resuspended in 300 μl of PBS. Samples were boiled for 10 min and then incubated for 2 h at 37°C with Zimolase (ICN) and for a further 2 h at 58°C with proteinase K (Sigma). Then, 200 μl of a detergent buffer were added and samples were boiled again for 10 min before addition of 1 ml of phenol:chloroform (Amersham) (1:1) and centrifuged at 20,000 × *g* for 20 min. 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 mixture. After storage of the samples overnight at −20°C, the DNA was then centrifuged at 20,000 × *g* for 20 min. Pellets were dried and resuspended in water. Real-time quantitative PCR was carried out using an ABI Prism 7000 thermocycler (Applied Biosystems), as previously described (30). The reaction mixture was prepared with 0.9 μM each oligonucleotide and 0.25 μM TaqMan probe in a final volume of 20 μl, to which 50 ng of DNA was added. The concentration of the DNA template was normalized with reference to previous PCRs with specific oligonucleotides in which DNA was denatured at 95°C for 10 min and amplified for 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR assays were carried out with oligonucleotides that amplify a region of the rRNA internal transcribed spacer genes. The oligonucleotides used were TaqMan 5′ TGAACCT GCGGAAGGATCAT, TaqMan 3′ ACGCAGCGAAATGCGATA, and TaqMan probe G-FAM (6-carboxyfluorescein)-TCAACAACGGATCTCTTGG-minor groove binder (MGB). Data were analyzed using SDS 7000 (1.1) software (ABI Prism).

Slot blot analyses. Different serum dilutions in Tris-buffered saline (200 μl each) were added to each well. Samples were blotted onto a 45-mm-pore-size nitrocellulose membrane (Bio-Rad) that had been previously hydrated in Tris-buffered saline for 10 min using a Bio-Dot SF apparatus (Bio-Rad). After blotting, the membrane was processed and developed as described above for Western blotting. The primary antibodies, rabbit polyclonal antibodies raised against *C. famata*, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *R. mucilaginosa*, or *S. cerevisiae*, were used at a 1:1,000 dilution. We used a donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham Biosciences) at a dilution of 1:5,000 as a secondary antibody.

RESULTS

Description of patients. Table 1 lists the patients involved in this study. Five individuals with diagnosis of AZOOR and five with diagnosis of SC were examined. These diagnoses were made on the basis of results of funduscopic examination, fluorescein angiograms, and campimetric tests. The results with respect to severity of visual defects are summarized in Table 1. AZOOR may represent an early stage in the evolution of the disease, ultimately leading to retinal necrosis.

Assays to analyze fungal infection. There is no universal test to determine whether disseminated fungal infection is present. In order to investigate this potential infection in patients diagnosed with AZOOR or SC, we carried out analyses using serum or whole peripheral blood by examination of the following: (i) fungal growth in appropriate media; (ii) antibodies against different *Candida* species (assayed by immunofluorescence); (iii) fungal genomes (examined using quantitative PCR); and (iv) antigens from *C. famata* and other yeasts (estimated by slot blot assays). Fungal growth was negative when 200 μl of serum or whole blood was seeded in liquid or agar YEPD media.

Analysis of *Candida* antibodies. The presence of antibodies against *C. famata*, *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* was assayed by immunofluorescence. Figure 1 illustrates the type of immunoreactions obtained with one AZOOR patient, one SC patient, and one healthy volunteer. There was clear immunoreactivity to some *Candida* species seen with the two patients, whereas immunoreaction was not observed with the healthy volunteer. Immunoreactivity varies according to the patient analyzed, but the results obtained with four out of five patients with AZOOR and four out of five patients with SC were positive in this test (Table 2). Not only the presence or absence of antibodies but also the *Candida* species recognized by the different serum samples differed from patient to patient. This variability may be dependent on the severity of the infection and the organs affected by the

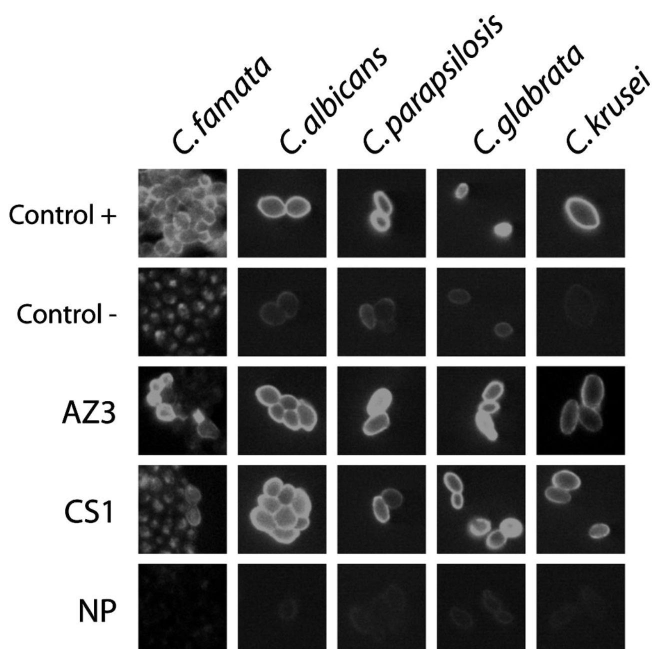

FIG. 1. Analysis by immunofluorescence to detect the presence of antibodies against different yeast species in serum samples from patients. In the case of *C. famata*, the protocol described in Materials and Methods was followed. As a positive control, rabbit antiserum against *C. famata* was employed; as a negative control, PBS, instead of the primary antibody, was added. For the remaining yeast species, a Euroimmun commercial kit was used and the controls were those provided by the commercial kit. Positive and negative controls for each species are shown in the corresponding columns. AZ3, AZOOR patient 3; SC1, serpiginous choroiditis patient 1; NP, healthy donor.

TABLE 2. Presence of antibodies against different *Candida* spp. in serum samples from patients[a]

Patient	Presence of indicated antibody				
	CF	CA	CP	CG	CK
AZ1	+++	−	−	−	−
AZ2	−	++	−	+	−
AZ3	++++	++++	++++	++++	+
AZ4	++++	++++	+	++++	+
AZ5	−	−	−	−	−
SC1	+	+++++	+	++++	+++
SC2	++++	++	++	+++	−
SC3	−	−	+	−	−
SC4	+	++++	−	+++++	+
SC5	−	++	++	+	−
Controls	−	−	−	−	−

[a] Percent positivity of fluorescence assay results compared to results obtained with the positive control provided by the manufacturer (Euroimmune test) was scored as follows: −, 0%; +, about 20%; ++, 40%; +++, 60%; ++++, 80%; +++++, 100%. As controls, serum samples from 12 healthy volunteers were tested. CF, *C. famata*; CA, *C. albicans*; CP, *C. parapsilosis*; CG, *C. glabrata*; CK, *C. krusei*.

disseminated candidiasis. It is also possible that the yeast species infecting each patient are different. By contrast, serum samples from 12 healthy volunteers were negative for the different *Candida* spp. tested (Table 2).

Fungal genomes in whole blood analyzed by quantitative PCR. Circulating fungal genomes in blood of patients with disseminated candidiasis have occasionally been observed (3, 9, 21, 22, 33). Whether such an observation is made probably depends on the activity of fungal foci present in the different organs or tissues. Even in cases in which viable cells were not detected in blood, it might have been possible to detect fungal DNA (21, 22, 27). Thus, for one AZOOR patient, yeast DNA was evidenced by quantitative PCR (Fig. 2A). Interestingly, four SC patients were also PCR positive. In one of them, there were 12,500 copies of rRNA genes per ml of blood (Fig. 2). On the other hand, the lack of genome amplification by PCR in samples from some patients may be because potential fungal infection was not reflected in blood at the time the sample was obtained.

Analysis of fungal antigens in blood serum. We have developed a very sensitive method based on the slot blot technique to analyze *C. famata* antigens in human serum samples (30). Figure 3A shows the type of reaction obtained with one AZOOR and one SC patient, whereas the test results obtained with serum from a healthy donor were negative. The analysis of serum samples from all patients with this method detected the presence of these antigens at moderate levels in samples from four of the AZOOR patients, but the results for the remaining patient were negative (Fig. 3B). The tests showed high amounts of *C. famata* antigen in two SC patients but low amounts in the other three. In such cases, it may be that antigens are not secreted into the bloodstream or that these antigens, if present, belong to fungal species not recognized by the rabbit antibody used.

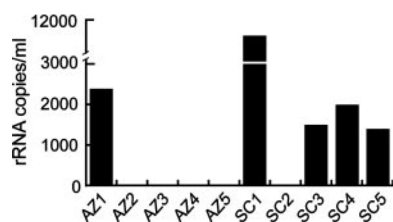

FIG. 2. Quantitative PCR analyses of whole blood. DNA was extracted from 200 μl of blood. PCR was carried out as described in Materials and Methods. Graphic shows the data obtained in numbers of fungal ribosomic RNA copies per milliliter of blood. NP1 and NP2 represent control serum samples from healthy donors. AZ1 to AZ5, AZOOR patients; SC1 to SC5, serpiginous choroiditis patients.

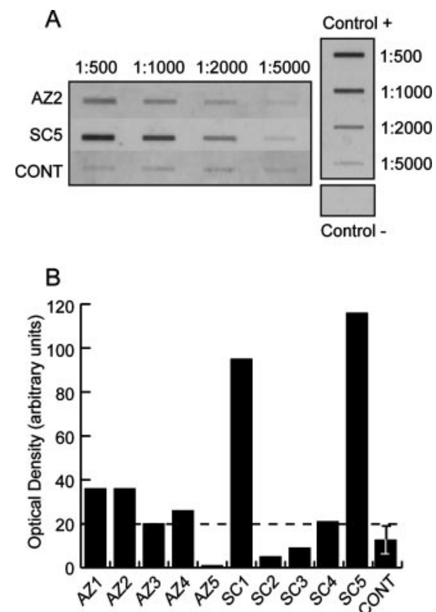

FIG. 3. (A) Presence of *C. famata* antigens in patient serum samples analyzed by slot blot. Different dilutions of serum samples were blotted onto a nitrocellulose membrane, which was incubated with the rabbit antiserum against *C. famata*. CONT, control sample using serum from a healthy donor; Control +, serum from a patient with chronic candidiasis; Control −, no human serum added. (B) Densitometric values of the 1,500 dilution from the slot blot analysis of serum samples from the indicated patients. The slot blot membrane was incubated with the rabbit antiserum raised against *C. famata*. CONT, controls. The values shown represent the median of results from 12 healthy volunteers. The bar indicates the standard deviation. AZ1 to AZ5, AZOOR patients; SC1 to SC5, serpiginous choroiditis patients.

To further investigate this possibility, rabbit antibodies were raised against other yeast species, namely, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *S. cerevisiae*, and *R. mucilaginosa*. These antibodies were used to assay for the presence of fungal antigens in the different serum samples. As shown in Table 3, no circulating antigens immunoreacted with *C. parapsilosis*, *S. cerevisiae*, or *R. mucilaginosa* antibodies in AZOOR patients. Only patient 3 presented moderate antigen levels that immunoreacted in the slot blot assay using *R. mucilaginosa* and *S. cerevisiae* antibodies. Notably, the serum sample from this patient (AZOOR 3) had a high titer of the *C. glabrata* antigen. The fact that some patients possess antigens that immunoreact with different antibodies may be due to cross-reactivity, but we cannot discard the possibility of mixed yeast infections. In the case of SC patients, no antigens that immunoreact with *S. cerevisiae* and *R. mucilaginosa* antibodies were found. Notably, SC patient 1 was positive for *C. albicans* antigens, whereas the levels of *C. parapsilosis* antigen in patient 4 and *C. glabrata*

TABLE 3. Immunoreactions of serum antigens with antibodies against different yeast species as analyzed by slot blotting[a]

Patient	Optical density (in arbitrary units)				
	CA	CP	CG	SC	RM
AZ1	19	4	17	3	0
AZ2	28	2	23	6	0
AZ3	11	3	117	10	15
AZ4	8	3	13	0	3
AZ5	0	2	0	0	0
SC1	57	3	4	4	0
SC2	4	5	11	2	0
SC3	2	4	0	0	2
SC4	10	18	7	0	3
SC5	1	5	0	0	1
Controls (median)	11.8 ± 6.7	7.8 ± 3.8	5.4 ± 1.4	1.4 ± 0.9	10 ± 4.0

[a] Different dilutions of sera were blotted onto a nitrocellulose membrane, which was incubated with rabbit antisera against different yeast species as described in Materials and Methods. Numbers represent optical density results obtained by densitometry of the band corresponding to a 1,500 dilution. For remarks concerning control patients, see footnote to Table 2. The control values correspond to medians and standard deviations. CF, *C. famata*; CA, *C. albicans*; CP, *C. parapsilosis*; CG, *C. glabrata*; SC, *S. cerevisiae*; RM, *R. mucilaginosa*.

TABLE 4. Summary of results obtained[a]

Patient	Antibody level	PCR result	Antigen level	Infection status
AZ1	Moderate	2,400	Low	Positive
AZ2	Moderate		Low	Positive
AZ3	High		High	Positive
AZ4	High		Low	Positive
AZ5	None		None	Negative
SC1	High	12,500	High	Positive
SC2	High		Uncertain	Uncertain
SC3	Low	1,500	None	Positive
SC4	High	2,000	Low	Positive
SC5	Moderate	1,400	High	Positive

[a] Antibody levels were scored according to the percentage of positivity of fluorescence assay results as follows (see footnote to Table 2 for explanation of symbols): ++++ or +++++, high; +++ or ++, moderate; +, low; −, none. PCR result values indicate numbers of rRNA gene copies per milliliter of blood. Antigen levels were considered high when the highest optical density value was 80 or higher; moderate when the highest value was between 80 and 50; low when the highest value was between 50 and 20; and uncertain when the highest value was between 20 and 10. "None" represents results for which the highest value was lower than 10.

antigen in patient 2 were low. In summary, apart from the presence of *C. famata* antigens in four AZOOR patients, the presence of high levels of *C. albicans* antigen in patient 1 and *C. glabrata* antigen in patient 3 should also be noted. SC patient 1 was positive for both *C. famata* and *C. albicans* antigens, whereas patient 5 only had *C. famata* antigens. Patient 2 had low levels of *C. glabrata* antigens only. No antigens immunoreacting with the different antibodies used were evidenced in SC patient 3. In principle, it may be possible that this patient was infected by fungal species other than the ones tested in our assay. A summary of all of the results is shown in Table 4.

DISCUSSION

Assessment of disseminated fungal infection cannot be easily incorporated into routine hospital analysis. In most cases, only fungal growth from different tissue samples is tested, but techniques of greater complexity must be implemented to detect this type of infection (1, 3, 10, 26, 31). However, these techniques are used only in cases of clinical suspicion of a fungal disease. Nonpathogenic yeasts such as *C. famata* do not provoke pain or fever, making the clinical diagnosis of such infection difficult. In addition, the viability of *C. famata* cells declines after incubation with human blood (our unpublished results). Therefore, simple analysis of yeast growth from blood samples may be misleading. In fact, in a case we reported recently involving an AZOOR patient, *C. famata* growth was found only twice over a 10-year period (6, 30).

On the other hand, the determination of the presence of antibodies against *Candida* species is not a reliable assay for detection of disseminated candidiasis (10, 32). Certainly, the presence of these antibodies suggests a yeast infection, but the contrary is not true. Patients infected with *C. famata* or other yeasts may be antibody negative for a number of reasons such as the severity of the infection and, most probably, the actual locations of the different fungal foci (7, 11, 32). One possibility to consider is that the presence of antibodies reflects past contact with *C. famata* and that antibody response persists after elimination of the yeast. This idea is not, however, supported by the finding that the antibody response to this yeast decreased when an AZOOR patient was treated with antifungal agents even though signs of infection were still apparent (30).

In our opinion, the presence of antibodies must be analyzed and the results should be contrasted with those of other tests. The most conclusive evidence for disseminated candidiasis comes from the detection of circulating fungal components in blood or other tissues, which should be free of microorganisms. The detection of fungal DNA in blood samples by PCR is the most conclusive evidence (1, 3, 10, 29, 42). However, circulating fungal DNA may be present in the bloodstream for only short periods of time. PCR analysis of vitreous liquid or choroid samples would be more conclusive, but these invasive techniques are more aggressive and riskier than a simple blood test and may cause discomfort to the patient. Indeed, a nonaggressive blood test analysis is preferable, and, to our knowledge, assaying for the presence of fungal antigens constitutes the most sensitive test currently available. This assay reveals the presence in serum of exported fungal proteins and those derived from yeast lysis. Patient serum sample results that are PCR negative could be positive in the slot blot assay (30).

Antigen analyses suffer from the drawback of low specificity, since the antibodies used would only recognize proteins from *C. famata* or cross-reactive species. This problem can be overcome, in part, by using a wide range of antibodies raised against different yeast species. This approach can also shed more light on the species of fungi that infect a given patient, as illustrated by the results seen with AZOOR patient 3, who showed high levels of *C. glabrata* antigens and moderate levels of antigens to other yeast species. Of interest, SC patient 5 exhibited high levels of antigens that reacted with rabbit polyclonal antibodies in the slot blot assay whereas this patient had no antibodies against *C. famata* and few against other *Candida* spp. One possible interpretation is that this patient was infected by *C. famata* or a close species that immunoreacts with the rabbit antibodies. On the other hand, the immune response

of this patient was poor and the human antibodies recognized *C. albicans* and *C. parapsilosis* with low efficiency. Testing for the presence of fungal polysaccharides [β-(1,3) glucans] in peripheral blood is not as reliable as other assays, since patients who are undergoing dialysis or who are following certain therapeutic treatments may give positive results (1, 15, 29). In conclusion, the clinician should consider the results from different tests on a case-by-case basis to determine the existence of infection.

Taking into account the considerations discussed above for the patients described in this study, there was evidence of yeast infection in four out of five AZOOR patients and in all SC patients. To ensure correct identification and treatment of this infection, long-term follow-up of these patients should be carried out. Analyses of blood samples taken at different times may provide further evidence of fungal infection, and eventually these analyses would serve as a means of monitoring the evolution of the disease in case antifungal treatment is implemented. The possibility that AZOOR and SC are caused by a yeast infection is reinforced by the known clinical data. Thus, these diseases are slow and progressive in development and are recurrent. Prolonged yeast growth may provoke tissue necrosis, leading to the appearance of scars and atrophy of the retina and choroid vessels. Notably, in such cases the eye fundus is pale and whitish in such cases, while in some cases, clear white dots appear. Evidence of strong fluorescence in fluorescein angiograms, typical of some of these patients, may reveal transient uptake of this dye by the fungus. In addition, similarities between AZOOR and histoplasmosis, a disease caused by the fungus *Histoplasma capsulatum*, with respect to the results of funduscopic examinations have been described previously (14, 17). Unambiguous confirmation that yeast infection causes AZOOR or SC will come from clinical trials that aim to investigate the beneficial effects of antifungal treatments. These trials face the problem of reaching high enough concentrations of the antifungal compound in the eye to inhibit *C. famata* or other fungi present. It is crucial to clarify the fungal etiology of both AZOOR and SC, since present treatments are sometimes based on the use of corticosteroids and other anti-inflammatory compounds (6, 13, 24, 28, 39). It is well established that corticosteroids increase susceptibility to fungal infections; indeed, fungal infections are more severe in mice treated with these compounds (25, 41). The presence of fungal infection in AZOOR or SC patients, even if not causative of disease, would be aggravated by corticosteroid therapy. Patients suffering from AZOOR or other related ocular diseases would benefit not only from a prompt diagnosis of a fungal infection but also, more importantly, from receiving adequate therapeutic treatment.

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