Evolution of antibody response and fungal antigens in the serum of a patient infected with *Candida famata*

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The presence of fungal antibodies and antigens in the serum of a patient diagnosed in 1996 with acute zonal occult outer retinopathy caused by *Candida famata* infection was examined. Antibodies against *C. famata* increased until 1999–2000 when antifungal treatment was initiated. The antibodies were detected by ELISA and immunofluorescence analysis using *C. famata*. These antibodies were not immunoreactive against several *Candida* species tested. Positive immunofluorescence was obtained with IgM, but not IgA, IgG or IgE. Moreover, the IgM response disappeared several months after treatment with antifungal compounds, despite the fact that *C. famata* antigens were present in the blood. Finally, a sensitive test was developed to assay for the presence of *C. famata* antigens in serum based on the immunodetection of fungal antigens transferred to a nitrocellulose membrane and incubated with rabbit antibodies raised against *C. famata*. According to this method, the infection diminished with antifungal treatment.

INTRODUCTION

The immunological response to fungal infections in humans is complex and still subject to much debate (Coleman *et al.*, 1998; d'Ostiani *et al.*, 2000; Gil, 1997; Lopez-Ribot *et al.*, 2004; Mencacci *et al.*, 1999; Pfaller, 1996; Shoham & Levitz, 2005). Cellular response, particularly macrophage function, may be crucial in combating systemic infections produced by different fungi (Badauy *et al.*, 2005; d'Ostiani *et al.*, 2000; Simitsopoulou & Roilides, 2005; Villar *et al.*, 2005). However, the importance of a robust antibody response to *Candida* infections has also been documented (Fernandez-Arenas *et al.*, 2004; Mencacci *et al.*, 1999; Witkin *et al.*, 1983). The fact that systemic candidiasis is much more common in immunocompromised patients points to a key role for the immune system in preventing this infection (Altamura *et al.*, 2001; Clemons *et al.*, 2000; Ruhnke, 2006; Sims *et al.*, 2005). Cellular immune response, together with the humoral response and innate immunity, are most likely necessary for efficient control of this type of systemic infection (Altamura *et al.*, 2001; Lopez-Ribot *et al.*, 2004). Although many studies have described the immune response in human candidiasis (Lopez-Ribot *et al.*, 2004; Mencacci *et al.*, 1999; Rozell *et al.*, 2006), hardly any have attempted to characterize the antibodies of patients infected with *Candida famata*.

Candida spp. account for a large percentage of human fungal infections. Apart from *Candida albicans*, which represents about 50 % of blood isolates (Canteros *et al.*, 1994; Peres-Bota *et al.*, 2004), other species such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* have been found in the blood of infected patients (Al-Hedaithy, 2003; Almirante *et al.*, 2006; Brandt *et al.*, 2000; Fanci *et al.*, 2005; Veldman *et al.*, 2006; Wagner *et al.*, 2005; Yang *et al.*, 2003). Viable *C. famata* has been found in about 1–2 % of patients suffering from systemic candidiasis (Al-Hedaithy, 2003; Canteros *et al.*, 1994; Krcmery & Barnes, 2002; Peres-Bota *et al.*, 2004; Pfaller *et al.*, 2003; Prinsloo *et al.*, 2003; Ruhnke, 2006; Tortorano *et al.*, 2006; Yamamoto *et al.*, 2002), but analysis of the presence of genomes by PCR yields a different percentage (Khan & Mustafa, 2001; Pryce *et al.*, 2003; van Deventer *et al.*, 1995). Although *C. famata* is considered to be non-pathogenic (Andrighetto *et al.*, 2000; Gardini *et al.*, 2001), it has been found in tissues as diverse as bone, blood and the CNS, and is associated with vision problems (Krcmery & Kunova, 2000; Prinsloo *et al.*, 2003; Rao *et al.*, 1991; St-Germain & Laverdiere, 1986). Recent evidence suggests that *C. famata* is the aetiological agent responsible for acute zonal occult outer retinopathy (AZOOR). We report here in detail the antibody response in an AZOOR patient described previously (Carrasco *et al.*, 2005). We also monitored the course of infection using different methods. Correct diagnosis of disseminated candidiasis is still elusive in some patients; thus different approaches are needed to demonstrate disseminated fungal

Abbreviation: AZOOR, acute zonal occult outer retinopathy.

infection (Ellepola & Morrison, 2005; Pontón, 2006; Yeo & Wong, 2002). In most hospitals, haemocultures are the main routine assay for detecting disseminated candidiasis (Ellepola & Morrison, 2005). However, unlike systemic candidiasis, viable *Candida* cells are seldom present in disseminated infections. PCR can overcome this problem but at least one fungal genome per assay must be present to determine the presence of yeast cells in blood (Bretagne & Costa, 2005). Other methods are based on detecting fungal metabolites or other components, such as proteins or polysaccharides, in blood serum (Ishibashi *et al.*, 2005; Mitsutake *et al.*, 1996; Pontón, 2006). Some of these methods detect specific proteins, such as glycolytic enzymes, heat-shock proteins or secreted proteases. The use of immunological assays to determine the presence of these proteins in human blood serum constitutes a more sensitive test for diagnosis of disseminated candidiasis. Diverse commercial kits have been developed to assay the presence of polysaccharides such as mannans or glucans in serum. We compared these techniques in serum samples taken at different times from a patient infected with *C. famata*. Our results suggest that analysis of fungal proteins in serum constitutes the most sensitive test to monitor the course of this infection.

METHODS

Yeast growth. The yeast was grown in YEPD medium (1% yeast extract, 2% peptone, 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 phosphate-buffered saline (PBS) containing 1 or 2 mg yeast after autoclaving and lyophilization. Each inoculum had been mixed previously with the same volume of Freund's adjuvant. Rabbits were inoculated up to four times and the antibody titre and specificity of the sera were tested by immunofluorescence and Western blotting.

Immunofluorescence and immunoelectron microscopy. For *C. famata*, 1 ml 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 in PBS/Tween 20 at 37 °C for 2 h, washed again with PBS/Tween 20 and incubated with the secondary antibody. Goat anti-human IgA, IgM and IgG (Sigma), or rabbit anti-IgG + IgA + IgM (Abcam), conjugated to fluorescein antibodies, were added at a 1 : 500 dilution; in some cases anti-IgG4 and IgE antibodies were used (a generous gift from M. Lombardero, ALK, Abelló, Madrid, Spain). In these cases, samples were then incubated with an anti-Ig fluorescein-conjugated antibody. The samples were incubated at 37 °C for another hour. 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 kit (Medizinische Labordiagnostika) was used in accordance with the manufacturer's instructions and using the same serum dilutions as for *C. famata*. For immunoelectron microscopy, we followed a protocol described elsewhere (Wright *et al.*, 1988), with some modifications: the cells were fixed for 2 h at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and washed after post-fixation

with 0.15% tannic acid in 0.05 M cacodylate buffer (pH 7.2). Cells were finally embedded in Epon (TAAB Laboratories). The sections were rehydrated in PBS and immersed in 0.5 M NH₄Cl for 15 min. Samples were then blocked with 1% BSA in PBS, incubated for 1 h with the primary antibody diluted in 1% low-fat dried milk in PBS at room temperature and subsequently washed with 0.1% BSA in PBS. After washing, secondary antibody conjugated to colloidal gold particles was added at a 1 : 10 000 dilution in PBS. Finally, samples were washed again with PBS and once again with triple-distilled water. The samples were analysed with a JEOL 1010 electron microscope.

ELISA. ELISA was carried out as described previously (Carrasco *et al.*, 2005). Briefly, a *C. famata* cell suspension diluted in PBS was seeded in ELISA microtitre plates (Maxisorp; Nunc). The plates were blocked with PBS containing 3% low-fat dried milk and 0.2% Tween 20. Serum from the AZOOR patient was added at a 1 : 200 dilution and plates were subsequently incubated with goat anti-human (heavy and light chain) Ig horseradish peroxidase-conjugated antibodies (Pierce) and washed five times. Colour development was accomplished by incubation with *o*-phenylenediamine (Sigma) and measured at 490 nm in a microplate reader (EL340; Bio-Tek Instruments).

Western blot assays. Yeast proteins were precipitated with trichloroacetic acid (10%) and fractionated by SDS-PAGE with 15% polyacrylamide, transferred to nitrocellulose membranes by wet immunotransfer and processed for Western blotting. Blocking, incubation with antibodies and washing were performed as described above for ELISA. Goat anti-human IgM horseradish peroxidase-conjugated antibodies (Amersham Biosciences) and an ECL kit (Amersham) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Agfa X-ray film.

PCR analyses. DNA was extracted from serum or whole blood. To this end, 200 µl serum was 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). A detergent buffer (200 µl) was added and samples were boiled again for 10 min before adding 1 ml phenol/chloroform (1 : 1; Amersham) and centrifuging at 20 000 g for 20 min. The upper aqueous phase was recovered and washed twice with ethyl ether. The DNA was precipitated by the addition of 3 vols absolute ethanol (−20 °C; Merck) to the aqueous phase. After storing the samples overnight at −20 °C, the DNA was centrifuged at 20 000 g for 20 min. Pellets were dried and resuspended in H₂O. The DNA preparations were incubated with oligonucleotides that hybridize to the rRNA genes (Li *et al.*, 2003; Nishikawa *et al.*, 1999) and amplify the first internal transcribed spacer region. The amplified product was analysed by agarose gel electrophoresis. The corresponding band was sequenced to determine unequivocally the presence of *C. famata* genomes. Real-time quantitative PCR was carried out in an ABI PRISM 7000 thermocycler (Applied Biosystems). The reaction mix was prepared with each oligonucleotide at 0.9 µM and 0.25 µM TaqMan probe in a final volume of 20 µl, to which 50 ng DNA was added. The concentration of DNA template was normalized against previous PCRs with specific oligonucleotides in which DNA was denatured at 95 °C for 10 min and amplified using 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Analysis of the data was carried out using SDS 7000 (v1.1) software.

Dot-blot analyses. Different serum dilutions (200 µl) in TBS were added to each well. Samples were blotted onto a 45 mm nitrocellulose membrane (Bio-Rad) previously hydrated in TBS 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. parapsilopsis*, *Rhodotorula mucilaginosa* or *Saccharomyces cerevisiae*, were used at a 1 : 1000

dilution. A donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham Biosciences) at a 1 : 5000 dilution was used as secondary antibody.

Detection of fungal polysaccharides. The presence of mannoses and β -1,3 glucan in serum was detected using the Platelia Candida AG test (Bio-Rad), as described by the manufacturer, or the Fungitell serum assay kit, performed by Fontlab (Barcelona, Spain) (Pazos *et al.*, 2005).

RESULTS AND DISCUSSION

The characteristics of our patient have been described previously (Carrasco *et al.*, 2005). Briefly, the patient was diagnosed with AZOOR in 1996 (aged 47). In 1999, *C. famata* was isolated from conjunctival exudate and blood. At the end of this year, intermittent antifungal treatment was administered as described previously (Carrasco *et al.*, 2005). Initially, the presence of anti-*C. famata* antibodies from serum obtained from different years was analysed by ELISA (Fig. 1a). The *C. famata* antibody titre increased until 1999. After administration of antifungal compounds, a clear decrease in the antibody titre was observed and it has remained low ever since. Analysis of these antibodies by immunofluorescence using *C. famata* also corroborated this observation (Fig. 1b). Thus positive immunofluorescence appeared with the sera obtained in June 1999, September 1999 and January 2000. Low reactivity was found with sera from June 1996 and August 2001, while no antibodies were detected from serum obtained in October 2005. The immunofluorescence observed with other yeast species was similar to that of the negative control without primary antibody. Only a slight reaction with *C. tropicalis* appeared, suggesting that the antibodies were fairly specific to *C. famata* in the immunofluorescence test. In our experience, some human sera do react with *C. tropicalis* in the Euroimmun test. Therefore, we consider this reactivity to be non-specific. Next, we wanted to analyse the type of antibody present in the positive serum from 1999. For this, a second anti-human antibody specific for IgA, IgM, IgG, IgG4 or IgE was employed after incubation of *C. famata* with this serum (Fig. 2). Interestingly, this assay revealed the presence of IgM but not IgG antibodies, even though the patient had been infected over several years. On the other hand, these IgM antibodies were not cross-reactive with *C. albicans* (Fig. 2). This serum was also analysed by Western blotting against *C. famata* proteins. Fig. 3(a) shows that there were two yeast proteins that gave a stronger reaction with serum from September 1999. These two proteins, of about 41 and 30 kDa, reacted more weakly with sera from October 2005, January 2006 and the control healthy donor. In addition, there were other proteins that immunoreacted non-specifically with all of the sera employed, such as a 47 kDa protein. Western blotting using *C. albicans* proteins revealed a low reactivity with the patient's serum, compared with a serum from a control patient infected with *C. albicans*. Moreover, no immunoreactivity was found against proteins from *Cryptococcus magnus* (Fig. 3a). Immunogold electron microscopy

Fig. 1. (a) ELISA to determine the presence of antibodies against *C. famata* in patient sera from different years. The assay was carried out as described in Methods. (b) Immunofluorescence analysis of different yeasts using different patient sera. In the case of *C. famata*, the protocol described in Methods was followed. As a positive control, rabbit antiserum against *C. famata* was employed; for the negative control, PBS instead of primary antibody was added. The Euroimmun kit for immunofluorescence was used for the remaining yeast species analysed. The controls were those provided by the kit.

analysis was carried out (Fig. 3b). This human antiserum clearly reacted with antigens at or in close proximity to the plasma membrane of *C. famata*. For comparison, a rabbit antiserum raised against this yeast was employed. It was noteworthy that both human and rabbit antisera recognized a similar structure close to the plasma membrane.

Fig. 2. Analysis of antibody type by immunofluorescence using serum from 1999. *C. famata* cells were treated as described in Methods and incubated with patient serum from June 1999. A range of anti-human antibodies specific for different immunoglobulins were employed as secondary antibody. As a negative control, PBS was added instead of primary antibody (not shown).

Fig. 3. (a) Left panel: Western blot of *C. famata* proteins with different sera from the patient or from a healthy donor (C-). Right panel: Western blot of *C. albicans* (C. alb) and *Cryptococcus magnus* (Cry) proteins incubated with the serum obtained from the patient in 1996. Ctrl C. alb, positive control of *C. albicans* proteins incubated with serum from a patient infected with this yeast. In all cases, the secondary antibody recognized IgM. Serum dilutions were at 1:4000, whereas the secondary antibody was used at 1:10 000 dilution. (b) Immunoelectron microscopy of *C. famata* cells. Yeast cells were incubated with patient serum (LC 1) or, as a control, with rabbit serum obtained against *C. famata* (D21), and then incubated with secondary antibody conjugated with colloidal gold. Magnifications are indicated.

As the patient's vision symptoms are not yet fully resolved, we tried to detect the presence of *C. famata* infection using PCR. DNA was extracted from serum and submitted to PCR using oligonucleotides that amplify the first intervening sequence between the rRNA genes, as described above. Real-time quantitative PCR using TaqMan probes was carried out with different samples (Fig. 4a). Evidence for the presence of *C. famata* genomes in the blood sera of this patient was found in the samples from October 1996 and June 1997, despite the fact that no viable yeast cells were recovered from these haemocultures. Furthermore, the presence of fungal genomes clearly diminished from June 1999, even though some symptoms of infection remained in this patient, such as the appearance of *C. famata* genomes in conjunctival exudates. The decrease in fungal genomes in blood from October 1996 probably corresponded to the discontinuation of immunosuppression therapy from the end of that year. These findings indicated that the presence of *C. famata* can be determined by PCR in samples that contain few antibodies and, conversely, that

Fig. 4. Detection of *C. famata* genomes by PCR. (a) Determination of the number of *C. famata* genomes in 200 µl serum by quantitative PCR, carried out as described in Methods. (b) PCR analysis of different serum samples obtained from the patient on the dates indicated above each lane. PCR assays were carried out with oligonucleotides that amplify a region of the rRNA gene internal transcribed spacer. C-PCR, negative PCR control without DNA; C+PCR, positive control of DNA extracted from *C. famata*; C-extraction, negative control of DNA extraction, prepared with phosphate buffer instead of a DNA sample. DNA size markers (bp) are indicated.

no fungal genomes are found at the peak of the antibody response. To confirm that the fungal genomes amplified in Fig. 4(a) were from *C. famata*, the amplified product obtained from another PCR was separated by agarose gel electrophoresis (Fig. 4b). A single product of about 240 nt was apparent in the serum from June 1997, which corresponded to the band obtained from *C. famata* DNA. This band was extracted and sequenced, revealing that it was indeed *C. famata*. Although no fungal genomes were detected by PCR from January 2000, abundant *C. famata* genomes were determined in a number of conjunctival exudates obtained up until May 2006 (data not shown).

As no *C. famata* genomes were detected in sera from recent years, we aimed to develop a system to detect the presence of fungal antigens in the blood serum. Our attempts to find fungal proteins by Western blotting using a rabbit antiserum raised against *C. famata* were unsuccessful. A much more sensitive test was the detection of proteins using a dot-blot assay. In this test, different serum dilutions were transferred to a nitrocellulose membrane and incubated with rabbit anti-*C. famata* antibodies. Using this assay, the presence of *C. famata* antigens was evident (Fig. 5a) and decreased over the years (Fig. 5b). This test has the advantage that it can be quantified by densitometry. Thus, with a 1 : 500 dilution, there was no immunoreactivity with sera from healthy donors (Fig. 5a). On the other hand, the patient serum did not react with rabbit pre-immune serum. Notably, some serum samples from the patient gave high test values, particularly before antifungal treatment was initiated. Despite prolonged administration of antifungal compounds, there was still evidence of fungal antigens using the dot-blot assay. Voriconazole treatment was initiated at the beginning of March 2006, but, curiously, there was an increase in the level of yeast antigens found in the blood, perhaps reflecting the action of this antifungal compound in tissues where infection persisted. For comparison, the same serum samples were tested for the presence of fungal polysaccharides (Ishibashi *et al.*, 2005). The Bio-Rad Platelia Candida AG test gave negative results in our samples (data not shown), whereas positive values were found when the Fungitell kit was employed. This latter test, which measures β -1,3 glucans, indicated the presence of this polysaccharide in the patient's blood over several years, but with large variations in the different samples assayed. The patient had become negative for this test from January 2006 but tested positive with the dot-blot assay. By comparison, the dot-blot assay was much more sensitive than the Fungitell test (Matthews & Burnie, 1998; Mitsutake *et al.*, 1996; Reboli, 1993), although both assays could be run to confirm the existence of fungal infection. Both assays could be useful for a first diagnosis, but follow-up of the patient should be carried out with the dot-blot test. Serum from a patient may even test negative for both assays, despite some residual infection remaining in some tissues of the body.

Finally, the specificity of the dot-blot assay was analysed employing several rabbit polyclonal antibodies raised

Fig. 5. (a) Dot-blot detection of *C. famata* antigens in patient sera. Different dilutions of sera were blotted onto nitrocellulose membrane and incubated with rabbit antiserum raised against *C. famata*. Rabbit pre-immune serum and sera from two healthy donors (controls 1 and 2) were used as controls. (b) Densitometric values of the 1 : 500 dilution from the dot blot of *C. famata* antigen in different serum samples obtained from the patient between 1996 and 2006. (c) Detection of fungal polysaccharides in different serum samples using a Fungitell kit. The dashed line indicates the minimum level of antigen necessary for the sample to be considered positive. Controls 1 and 2 were sera from healthy donors.

against different yeast species (*C. albicans*, *C. parapsilosis*, *C. glabrata*, *R. mucilaginosa* and *S. cerevisiae*). The potency and specificity of these antibodies were estimated using immunofluorescence and Western blot assays against the corresponding yeasts. Specific reactivity of the serum was exhibited against *C. famata* antibodies, whereas partial cross-reactivity was found with rabbit antibodies raised against other yeasts but not with the anti-*S. cerevisiae* antibodies (Fig. 6). In conclusion, these findings indicated that patient serum contains antigens that are preferentially recognized by *C. famata* antibodies and that partially cross-react with antibodies against other related yeast species.

Fig. 6. Dot-blot analysis of the patient serum using rabbit antibodies raised against different yeasts. (a) Dilutions from 1 : 200 to 1 : 5000 of the serum obtained from the patient in June 1996 were blotted and the nitrocellulose membrane was incubated with different antisera raised against the indicated species, obtained as described in Methods. (b) Densitometric values of the 1 : 500 dilutions shown in (a).

In this work, we have examined in detail one patient infected with *C. famata*. In order to generalize the findings reported, more patients should be studied. Little is known about the human immune response to disseminated infection by *C. famata*. In principle, this yeast has been classified as non-pathogenic and so does not produce an overt inflammatory reaction. *C. famata* may use different strategies to evade the immune response, one of them being the production of superoxide dismutase, which interferes with macrophage function (Garcia-Gonzalez & Ochoa, 1999). Other strategies common to other yeasts may be the production of a mucous environment, as well as precipitation of calcium carbonate at the sites of infection as a result of the drop in pH associated with fungal metabolic activity. In the patient studied here, the immune response with regard to antibody production was rather weak. The IgM response quickly disappeared after several months of antifungal treatment, even though the infection still remained in the periocular mucosa and probably in the retina and optic nerve. The cellular immune response of

this patient measured by the presence of specific T-lymphocytes was also low, despite the fact that he was not immunocompromised and was healthy apart from the vision problems. The rather low stimulation of the immune system observed in this patient thus may be one of the reasons for the prolonged infection described. Stimulation of the immune system certainly may help to combat and eventually eradicate this infection (Lopez-Ribot *et al.*, 2004).

From the point of view of diagnosis, we developed and compared different approaches to detect *C. famata* infection. Although the presence of the antibody response should be assayed, as in other *Candida* infections, this test is of limited diagnostic value (Quindos *et al.*, 2004). It must be stressed that the presence of viable *Candida* in blood was rarely detected in our patient. Once again, although haemoculture should be done, the absence of yeast growth does not rule out the possibility of a systemic or disseminated infection (Einsele *et al.*, 1997). The use of PCR is of greater value. Although this technique is very sensitive and we can detect a single genome copy in our assays, fungal genomes may not be circulating in the blood, i.e. the yeast may be located at foci in different tissues without any cells, either dead or alive, entering the bloodstream (Khan & Mustafa, 2001). Under these conditions, it is possible that the synthesis of polysaccharides may occur or export proteins could reach the blood and these could be detected in the serum (Mitsutake *et al.*, 1996; Pontón, 2006). In this regard, the system developed to analyse *C. famata* antigens by dot blot using a rabbit antiserum was of special interest. This assay was highly sensitive, as the presence of these antigens could be detected at a 1 : 500 or even a 1 : 1000 dilution. With this test, we could monitor the efficacy of the different antifungal treatments. The amount of protein detected by this method was in the femtomolar range, which is below the limit of detection of Western blotting.

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