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Detection of Antibody against Fungal Glucosylceramide in Immunocompromised Patients: A Potential New Diagnostic Approach for Cryptococcosis

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Abstract

We have developed an ELISA to determine the value of anti-glucosylceramide antibody for the prediction of disseminated cryptococcosis in immunocompromised subjects and performed a clinical prospective study at the Medical University of South Carolina. The study enrolled a total of 53 patients who were free of active fungal diseases at the time of enrollment but at risk of developing one because they were all immunocompromised, *e.g.*, (1) patients positive for HIV and (2) patients post- or awaiting solid organ transplantation. Among 53 patients enrolled, two patients developed invasive cryptococcosis, and in both patients, IgM anti-GlcCer was detected in sera using the ELISA at least 6 weeks prior to the clinical presentation of the brain disease. These results were corroborated by a cryptococcal antigen lateral flow assay, which was also positive in serum prior to the development of meningoencephalitis. However, a high number of positive results were also detected in patients with no evidence of cryptococcosis. This study highlights the

potential utility of this new assay in early diagnostic testing algorithms for patients at risk for cryptococcosis, but further investigations are needed to validate the sensitivity and specificity of the glucosylceramide ELISA as a predictor of cryptococcosis.

Keywords

Glucosylceramide; Antibody; ELISA; HIV; Cryptococcosis; Fungal infection

Introduction

Invasive fungal infections, such as cryptococcosis, candidiasis and aspergillosis, have dramatically increased in the last two decades and are associated with very poor prognosis [1]. Although significant progress has been made to improve molecular and genetic tools to study their development and progression, studies to provide better tools for an early diagnosis are urgently needed (reviewed in [2]). Our laboratory has focused on understanding the molecular mechanisms of fungal pathogenesis, particularly the role of bioactive lipids in this process, both at a mechanistic and translational level. We discovered that the sphingolipid pathway represents a rich reservoir for signaling molecules [3]. In particular, the reactions regulating complex sphingolipids such as inositol phosphoryl ceramide (IPC) impact virulence at several levels (reviewed in [4]). Additionally, the complex sphingolipid glucosylceramide (GlcCer) plays a crucial role in the survival of *Cryptococcus neoformans* (*Cn*) in the extracellular environment such as alveolar spaces. Specifically, GlcCer is required for *Cn* to cause disease once the fungus is introduced intranasally [5]. As a result, mice infected with mutant cells lacking the enzyme that forms GlcCer (*gcs1*) do not die of *Cn* infection, and this lack of pathogenicity has been traced to the formation of granulomas, which contain fungal cells in the lung. Thus, the production of GlcCer is required for the fungus to leave the lung and disseminate to the bloodstream and reach the brain. GlcCer is also required for the pathogenicity of other fungal pathogens [6–9].

The host (both mice and humans) responds to the *Cn* infection with the production of antibody against GlcCer [10], and administration of monoclonal antibody against GlcCer protects mice against lethal cryptococcal infection [11]. Interestingly, following intranasal infection of mice with 10^3 wild-type *Cn* cells, we detected IgM antibodies in the bloodstream prior to the dissemination of fungal cells to the brain [12]. Thus, we hypothesize that the detection of serum IgM against fungal GlcCer has future potential as an early diagnostic method of cryptococcosis. To investigate this, we analyzed serum samples of immunocompromised patients for the presence of anti-GlcCer antibody and evaluated the titer in the context of the clinical manifestations and other signs of *Cn* dissemination.

Materials and Methods

Patient Population

Fifty-three (53) patients were enrolled in the clinical study which ran at the Medical University of South Carolina (MUSC) from January 2009 to January 2011. The sample

population comprised the following groups: patients positive for HIV as well as patients who had undergone solid organ transplantation. At the time of enrollment, patients signed a consent document approved by the Institutional Review Board of MUSC to provide 5 ml of blood every 1–3 months. The collected blood was then sent to our laboratory for analysis. Sera was obtained by spinning blood at $3,000\times g$ for 10 min at 4°C and stored at –80°C until used. When diagnosed with cryptococcal meningitis, patients were sampled weekly where possible. Each patient was compensated per sample.

ELISA Assay

The assay was performed in a 96-well microtiter plate (Maxisorp NUNC). First, the wells were coated with 50 µl/well of 160 µg/ml of soy glucosylceramide (Avanti Polar Lipids, Inc., Cat # 131304) in methanol and incubated overnight at 4°C. The plate was then blocked with 5% BSA in phosphate-buffered saline (PBS), incubated 1 h at 37°C and then washed three times with PBS/0.1% Tween 20 (PBST). Serum samples at a dilution of 1:32 in PBS were added and incubated 1 h at 37°. The positive control, α -GlcCer IgM [12] (0.8 mg/ml) was also diluted 1:32 in PBS. After three washes with PBST, the plate was incubated with 50 µl per well of either goat anti-human IgM-HRP (μ -chain specific) (Sigma Cat # A0420) diluted 1:50,000 with 1% BSA/PBS or goat anti-mouse secondary IgM (μ -chain specific) (Sigma Cat # A8786) diluted 1:30,000 with 1% BSA/PBS, for 1 h at 37°C. After three washes with PBST, the color was developed with 50 µl/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma Cat # T0440). The reaction was then stopped with 50 µl 2 M H₂SO₄ and the plate read at 450 nm with a VersaMax plate reader.

Cryptococcal Antigen (CrAg) Lateral Flow Assay

This assay is an immunochromatographic test system for the qualitative detection of capsular polysaccharide antigens of *Cn* in serum and cerebrospinal fluid (CSF). The strips for this test were obtained from Immuno Mycologics, Inc (IMMY; Norman, Oklahoma), and the test was performed according to the manufacturer's directions at a dilution of 1:2, except for patient 23 whose samples were analyzed at a dilution of 1:2048. The presence of two lines, corresponding to Test and Control, indicates a positive result. A single control line indicates a negative result.

Results and Discussion

Over a period of 2 years, it was anticipated that patients would be enrolled at MUSC from five groups for the study: (1) patients positive for HIV (HIV+) (2) recipients of solid organ transplantation (SOT) (3) patients with lympho-proliferative disorders including leukemia and lymphoma (4) recipients of bone marrow transplantation and (5) patients immunosuppressed or receiving long-term immunosuppressive therapy for conditions such as sarcoidosis and connective tissue diseases. It was imperative that none of the patients enrolled in the study had any prior history of cryptococcosis or any other active fungal infections at the time of enrollment. While neutropenic patients are usually not at particular risk for developing cryptococcal infection, patients receiving immunosuppressive therapy and AIDS patients are more susceptible to cryptococcosis due to low (<50) CD4 T cell counts [13, 14]. A count less than 200 indicates the immune system is severely weakened,

and the HIV+ person is at a much greater risk of opportunistic infections [15]. However, despite low levels of serum immunoglobulins in immunocompromised patients, normal or near normal antibody production has been observed [16, 17].

Actual enrollment numbers (Fig. 1) highlight the difficulty of patient enrollment in groups other than (1) and (2) due to logistical difficulties of gaining access to patients in other clinics beyond the infectious disease clinic. While clinicians were willing to allow recruitment from the other patient populations, conflicts with clinic space and scheduling impaired the ability to recruit from other groups. 79% of patients were HIV+, while the remaining 21% were patients who had received SOT, predominantly kidney or liver transplants (Fig. 1a). This latter group was also predominantly Caucasian. The breakdown of racial categories over both groups was as follows: male Caucasian 19%, male African–American 53%, male Asian 2%, female Caucasian 6% and female African–American 20% (Fig. 1b). These numbers can be considered to be somewhat representative of the HIV+ and transplantation communities in Charleston, South Carolina, but actual numbers, particularly among the African–American community, may be greater. All the patients in the HIV+ group had CD4 counts less than 200 except patients 16 and 18, whose CD4 counts were 220 and 292 counts, respectively, on enrollment. Those patients whose viral loads were <40 or not detected (nd) copies per milliliter had been compliant with HAART.

For the ELISA, samples were diluted 1:32 in PBS. Absorbance cutoff signifying a positive IgM anti-GlcCer reading was set at 0.200 AU based on data from our previous studies in mice [12], as this mammalian model was considered a “true” negative control in being free of any previous (asymptomatic or symptomatic) fungal infections. In fact, since GlcCer is produced by a variety of fungi, such as *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and several dimorphic fungi (reviewed in [18], and [19]) it is expected that humans may develop IgM anti-GlcCer also when infected by these fungi.

In our study, out of the fifty-three enrolled patients (Fig. 2), thirty were identified as positive for IgM anti-GlcCer from the ELISA, while sixteen were negative and seven were lost to follow-up (Fig. 2), often right after the enrollment visit. Of the thirty positive for IgM anti-GlcCer, twenty-one had a positive IgM anti-GlcCer reading for 2–6 months via the ELISA, whereas nine started showing a positive IgM anti-GlcCer value only in the last couple of months of the study. For these patients in the latter group, it remains to be seen whether they will develop a fungal disease. Of the twenty-one positive for IgM anti-GlcCer, two were diagnosed with cryptococcal meningitis and we will discuss these two patients in greater detail. Given the high number of positives, we examined further why this was the case and found three of these patients, predominantly male, had a history of oral candidiasis. For the other sixteen positive patients, we could not identify any previous fungal infections. However, all sixteen of these patients showed a relatively low positiveness (*i.e.*, absorbance values between 0.200 and 0.400 AU), compared to the two patients who were diagnosed with cryptococcal meningitis (AU 0.300–0.700) suggesting that the test needs a better standardization with a much larger group.

As mentioned, two patients (patient 23 and patient 11) were diagnosed with cryptococcal meningitis during the course of this study and their IgM anti-GlcCer results from the ELISA

correlated well with the progression of disease, especially for the patient who was compliant with his highly active antiretroviral therapy (HAART) outside of the hospital setting.

Patient 23 is a 62-year-old African–American male who joined the study in August 2009. He has a history of HIV, hepatitis, candidal esophagitis and chronic alcohol abuse. He presented with severe headache on visit 3 (December 2009), which had been ongoing for 2 weeks, with occasional nausea and vomiting. At that time, he tested positive for cryptococcal antigen in his cerebrospinal fluid and had an absolute CD4 count of only 2 (normal values are 500–1400) (Table 1). In fact, his CD4 counts were extremely low for the duration of this study, even though he was on HAART during this period. While his CD4 numbers did improve, they were still significantly below normal values. The patient's white blood cell counts remained in the normal range (4.800–10.800 K), when he remained compliant with his HAART regimen, as well as amphotericin B and flucytosine. During the compliancy period, the IgM anti-GlcCer results remained below the positivity cutoff of 0.200 AU. This continued for almost a year (Table 1). However, it is possible that the patient had already developed an asymptomatic *Cn* lung infection at the time of enrollment, given the positive results of the IgM anti-GlcCer at enrollment and early sampling points. The CrAg assay results were positive for all the serum samples obtained for patient 23, suggesting again that either the patient had a low titer of capsular material in the bloodstream throughout the observation period and/or the CrAg assay may have issues of cross-reactivity. Studies are underway to study this potential complication against several *Candida* species, as well as *Mycobacterium tuberculosis*, *Pneumocystis carinii*, hepatitis A and C viruses, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Thus, in this patient, the ELISA results overall trended well with severity of symptoms. Unfortunately, his viral load and IgM anti-GlcCer readings had gone back up at the end of the study when he was no longer compliant with HAART and/or with his antifungal maintenance therapy.

Patient 11 is a 32-year-old African–American male who was in the study since its commencement in January 2009. He has a history of HIV/AIDS and no record of any previous fungal infection. He presented with positive serum cryptococcal antigen test at visit 7 (December 2009; Table 2) during the study after he had attended and been tested at an infectious disease clinic. At this time, he had endorsed a 2-week history of calvarial headaches, nausea and vomiting, as well as fevers and chills, and blurry vision. He was discharged following treatment with the diagnoses of cryptococcal meningitis, oral candidiasis, acute renal failure and AIDS. He had been on HAART regimen since July 2009, his CD4 counts were low (11), and his WBC count was at the lower end of the normal range, fluctuating between 4.5 and 5 K.

We found that all samples but the first three collected from patient 11 were positive for CrAg (Table 2). Interestingly, also IgM anti-GlcCer antibody was negative on the first three samples and became negative again on visit 9 and 23. His viral load remained high and CD4 counts remained low throughout the study further suggesting the lack of compliance to HAART.

Interestingly though, the IgM anti-GlcCer (Table 2) does not show a definitive trend as was observed for patient 23, as patient 11 did not remain compliant with his anti-retroviral

regimen or his antifungal maintenance therapy over the course of the clinical study. This may suggest that a negativization of the IgM anti-GlcCer value may be a useful indicator that the patient is compliant and responding to the therapy.

Unfortunately, several patients were positive for IgM anti-GlcCer antibody even if they were “clinically free” of cryptococcosis or any other fungal infection, suggesting that this test has a very low specificity and a low negative-predictive value. None of the patients enrolled in our study who remained free of cryptococcal meningitis showed positive CrAg assay results. Thus, the overall utility of the IgM anti-GlcCer ELISA as a potential diagnostic method, while it reliably predicted the onset of cryptococcal meningitis in two patients, is hampered by the fact that it was highly positive in patients with no signs or symptoms of active fungal infections. This may be due to cross-reactivity issues, and plans are underway to use the ELISA for the detection of IgM anti-GlcCer antibody against several other fungal and bacterial pathogens. Alternatively, the absorbance cutoff value of 0.200 AU could be increased once the test has been standardized with a much larger group of patients to get a true cutoff value in humans.

In conclusion, the IgM anti-GlcCer assay reliably predicted the onset of cryptococcal meningitis in two patients, in which the IgM antibody anti-GlcCer appears to correlate well with the severity of the cryptococcal disease. Clearly, additional cases need to be evaluated in order to assess whether this method could be a predictor and/or a prognostic factor of cryptococcosis or other fungal diseases, given the high number of patients who tested positive in the ELISA despite having no evidence of cryptococcosis.

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Fig. 1. Breakdown of patients enrolled in the clinical study at MUSC. **a** Patients were enrolled from 2 groups: HIV-positive (HIV+) and solid organ transplantation (SOT). **b** Racial breakdown of patients representing the immunocompromised population in Charleston SC

Fig. 2.

Flowchart showing that 4% of patients enrolled in the study developed cryptococcal meningitis; however, several other patients had positive results in the ELISA, indicating the need for further validation of the assay

IgM anti-GluCer results, CrAg assay data, CD4 counts and white blood cell (WBC) counts for patient 23 where CD4 and WBC counts are missing, and these were not available on the day of the blood draw

Table 1

Visit number	Date	IgM anti-GluCer (abs 450 nm)	CrAg result	CD4/CUMM	HIV IRNA-viral load (copies/ml)	WBC count (K)
1	8/19/2009	0.294	+	6	73003	3.87
2	11/23/2009	0.391	+	4	29379	3.42
3	12/11/2009	0.39	+	2	3437	6.31
4	12/22/2009	0.155	+			6.43
5	12/31/2009	0.18	+		<40	
6	1/15/2010	0.169	+			
7	3/15/2010	0.147	+			3.05
8	4/28/2010	0.129	+	72	0	
9	10/20/2010	0.137	+	55	274	3.91
10	1/19/2011	0.45	+	27	42460	3.12

Table 2

IgM anti-GluCer results, CrAg assay data, CD4 counts and white blood cell (WBC) counts for patient 11 where CD4 and WBC counts are missing, and these were not available on the day of the blood draw

Visit number	Date	IgM anti-GluCer (abs 450 nm)	CrAg result	CD4/CUMM	HIV 1RNA-viral load (copies/ml)	WBC count (K)
1	1/13/2009	0.12	-			2.53
2	4/27/2009	0.048	-			
3	7/22/2009	0.057	-			4.92
4	9/25/2009	0.067	+			
5	10/26/2009	0.464	+	12	80994	5
6	11/30/2009	0.372	+			
7	12/14/2009	0.68	+	11		4.78
8	12/21/2009	0.344	+			4.49
9	12/30/2009	0.13	+			3.78
10	1/6/2010	0.392	+			
11	1/20/2010	0.461	+			
12	1/27/2010	0.295	+			
13	2/1/2010	0.307	+			
14	2/10/2010	0.652	+	6	56116	3.5
15	2/17/2010	0.516	+			
16	2/26/2010	0.278	+			
17	3/31/2010	0.491	+			
18	4/30/2010	0.294	+	5	67269	2.9
19	5/26/2010	0.41	+			
20	6/21/2010	0.291	+			
21	7/22/2010	0.392	+			
22	9/20/2010	0.469	+	5	40845	3.93
23	10/20/2010	0.187	+			
24	11/22/2010	0.393	+			
25	12/28/2010	0.524	+	5	118897	3.12
26	1/31/2011	0.384	+			