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BRIEF REPORT

Disseminated Cryptococcosis Following Eculizumab Therapy: Insight Into Pathogenesis

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Eculizumab, a recombinant humanized monoclonal antibody (mAb), is used for the treatment of patients (both adults and children) with paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. This mAb binds to complement protein 5 (C5), thereby inhibiting its cleavage. On the other hand, one of the C5 cleavage products, C5a, is a potent anaphylatoxin with proinflammatory properties, involved in antimicrobial surveillance. Administration of eculizumab has been reported to make patients more susceptible to infection by encapsulated bacteria. Here, we are reporting an adult case of disseminated infection due to the encapsulated yeast *Cryptococcus neoformans* following eculizumab therapy and discuss its pathogenesis.

Keywords. atypical hemolytic uremic syndrome; aHUS; complement component c5; cryptococcosis; eculizumab; pathogenesis.

The complement system is an integral part of humoral immunity, the activation of which can lead to direct microbicidal activity or opsonization of invading pathogens facilitating their phagocytosis. The complement activation also results in the release of potent anaphylatoxins including C5a, which is a proinflammatory mediator [1]. Cytokine production and antimicrobial activity are influenced by C5a [2]. However, the harmful anaphylatoxin effect due to excess production of C5a, an uncontrolled host inflammatory response, facilitates infections caused by encapsulated bacteria [3].

The role of the host complement system in the defense against fungal pathogens is insufficiently understood. The presence of a cell wall in fungi avoids direct fungicidal activity of the complement system and invasive fungal pathogens have developed strategies to escape complement attack [4]. *Cryptococcus neoformans* is an encapsulated yeast that causes disseminated infection in patients with cellular immunodeficiency. This yeast is also a potential activator of the complement system [5]. The importance of C5 against *C neoformans* infection has been demonstrated in an animal model [6].

Eculizumab, a recombinant humanized monoclonal IgG2/4k antibody that prevents terminal complement activation by binding to C5 [7], is now widely used in clinical medicine [8]. Knowing its mechanism of action, eculizumab therapy nonsurprisingly increased the susceptibility of patients for encapsulated bacterial infections. Until recently, there was no reports suggesting other infectious complications. Since then, a limited number of case reports of invasive fungal infections following eculizumab therapy has been described [9, 10]. Here we report an adult patient developing *C neoformans* fungemia following eculizumab therapy and propose insight into pathogenicity related to C5.

CASE REPORT

A 58-year-old man had a recent history of atypical hemolytic uremic syndrome (aHUS) requiring transient extrarenal epuration. For aHUS treatment, he received eculizumab (900 mg) daily between 23 and 27 April 2021 (4 pulses) and a fifth pulse on 27 May 2021, with a prophylaxis by oral penicillin. A new episode of heart failure associated with unexplained fever (38.5°C on peak) prompted us to perform blood cultures starting from 14 July 2021. Fungemia due to *C neoformans* (identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry) was evidenced on blood cultures performed on 14 and 15 July. Following the diagnosis of fungemia, an exhaustive assessment of potential dissemination was performed immediately. Fungal cultures of his cerebrospinal fluid (CSF), bronchoalveolar lavage, skin, urine samples, and subsequent blood cultures from 16 July onward were negative. His serum and CSF were negative for cryptococcal antigen (lateral flow assay; IMMY). Complementary biological investigations (human immunodeficiency virus [HIV] antibodies, anti–granulocyte-macrophage colony-stimulating factor, and anti–interferon gamma [IFN-γ] antibodies) were also negative. Fluconazole (400–800 mg/day, according to renal function) was initiated immediately when fungemia was diagnosed.

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During July–September 2021, his CD4, CD8, and neutrophil counts were 162–230 cells/μL, 88 cells/μL, and 6300–6900 cells/μL, respectively. In February 2022, his CD4, CD8, and neutrophil counts were 253 cells/μL, 146 cells/μL, and 5300 cells/μL, respectively. In July 2022, fluconazole was stopped and as of February 2023, the patient remains healthy.

METHODS

Complement Assay

A functional complement activity assay of the classical (CH50) hemolytic activity was determined according to standard procedures. C3 and C4 plasma levels were measured by nephelometry (Siemens, Newark, Delaware), and levels of alternative pathway (factor H [FH], factor I [FI]) by in-house enzyme-linked immunosorbent assay (ELISA). Anti-FH antibodies were screened as reported [11]. Soluble C5b-9 level was determined using MicroVue sC5b-9 Plus enzyme immunoassay kit (Quidel). Screening for variants/complex rearrangements in FH, FI, membrane-cofactor protein, C3, and factor B genes was performed using next-generation sequencing and multiplex ligation-dependent probe amplification [12]. Plasma C5 level was determined by ELISA using nonbiotinylated/biotinylated anti-C5 antibodies, streptavidin-horseradish peroxidase and the substrate 3,3′,5,5′-tetramethylbenzidine; C5a was estimated by ELISA using human C5a detection kit (DuoSet, R&D Systems).

Immune Cell Isolation and Stimulation With *C neoformans*

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by a density-gradient separation using Ficoll (Eurobio, France). PBMCs were resuspended in RPMI (Gibco) supplemented with 10% pooled normal human serum (NHS) or autologous serum (AuS) and seeded (2×10^6/mL, 100 μL/well) in 96-well culture plates (Nunc Labware, Sigma-Aldrich). Neutrophils were isolated from whole blood using EasySep isolation kit (Stemcell Technologies), suspended in RPMI supplemented with NHS or AuS (1%), and seeded (1×10^6/well) into 96-well plates. *Cryptococcus neoformans* (H99) was cultured in yeast extract–potato dextrose medium for 24 hours, harvested, and washed with water. To PBMCs or neutrophils, *C neoformans* yeasts suspended in RPMI supplemented with NHS or AuS (10% for PBMCs, 1% for neutrophils) were added (multiplicity of infection 1:10; 100 μL/well) and incubated at 37°C with 5% carbon dioxide for 24 hours. Collected culture supernatants were analyzed for cytokines using ELISA kits (R&D Systems). The statistical analysis was performed by 1-way analysis of variance (Tukey multiple comparison test).

C5 Cleavage Activity of *C neoformans*

Three micrograms of human C5 (Merck) was incubated with 1×10^6 *C neoformans* (H99 strain or patient's isolate) in gelatin veronal buffer supplemented with Ca^{+2}-Mg^{+2} (total volume, 100 μL). The aliquot was taken for C5a measurement by ELISA (R&D Systems). Assay was also performed with heat-killed *C neoformans*. *C neoformans* (H99, 1×10^7) was cultured in collagen medium (10 mL) for 24 hours at 37°C. The culture supernatant was concentrated to 1 mL, from which 20 μL was added to C3, C4, or C5 (3 μg); volume was made to 50 μL with phosphate-buffered saline and incubated at 37°C for 1 hour. The reaction mixture (25 μL) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4%–12% gradient gel, ThermoFisher Scientific); protein bands were visualized by Coomassie blue staining.

Consent Statement

The patient gave written consent to be a part of this study. Healthy donors' blood samples were obtained from the Etablissement Français du Sang (Paris, France) with written informed consent as per the guidelines provided by the institutional review board of Institut Pasteur, and used to separate sera.

RESULTS

Complement Assay

At the time of aHUS diagnosis (April 2021), the patient presented with alternative pathway consumption (low C3), levels of FH and FI were within normal ranges, and the screening for anti-FH antibody was negative (Figure 1*A*). CH50 blockage was complete after the first dose of eculizumab (CH50 <10%). In July 2021, CH50 was within the normal ranges showing the absence of C5 cleavage blockage. Screening for variants in complement genes associated to aHUS was negative.

C5/C5a Level in Plasma and the Immune Response

Levels of serum C5a were significantly low during eculizumab therapy, compared to C5a levels in healthy donors, suggesting inhibition of C5 cleavage upon therapy. Two months after the last eculizumab pulse, there was an increasing trend in the serum C5a level (Figure 1*B*); this trend was opposite for plasma C5 (Figure 1*C*). PBMCs and neutrophils isolated from this patient were made to interact with *C neoformans* in presence of NHS or AuS. Although there were no significant differences in the tumor necrosis factor alpha (TNF-α), interleukin (IL)–1β, and IL-6 levels, both PBMCs and neutrophils stimulated with *C neoformans* in medium containing AuS showed significant increase in the IL-8 levels compared to the medium containing NHS (Figure 1*D* and 1*E*).

C neoformans Specifically Cleaves C5

In vitro, when C5 was incubated with *C neoformans* (H99), there was a time-dependent increase in the C5a released (Figure 2*A*), but heat-killed *C neoformans* failed to cleave C5 (Figure 2*B*). We then cultured *C neoformans* in collagen medium (as airborne *C neoformans* encounters collagen in the airway), collected the culture supernatant, and made it to interact with complement proteins C3b, C4b, and C5b. Interestingly, the culture supernatant showed only C5b-degrading activity; specifically, there was

A

Sampling date	21/04/2021	24/04/2021	28/04/2021	05/05/2021	11/05/2021	01/07/2021	20/07/2021	21/02/2022	21/02/2022
Complement factor									
CH50	34	<10	73	67	29	98	69	126	130
C3	552	842	1070	1150	1010	1190	904	1210	1190
C4	109	183	247	332	281	461	317	355	350
sC5b9	1058	ND	ND	ND	ND	224	ND	ND	ND
FI	172	ND	ND	ND	ND	154	ND	ND	ND
FH	134	ND	ND	ND	ND	123	ND	ND	ND
Ac-anti FH	Neg	ND	ND	ND	ND	Neg	ND	ND	ND
Ac-anti FB	Neg	ND	ND	ND	ND	ND	ND	ND	ND
Ac-anti C3b	Neg	ND	ND	ND	ND	ND	ND	ND	ND

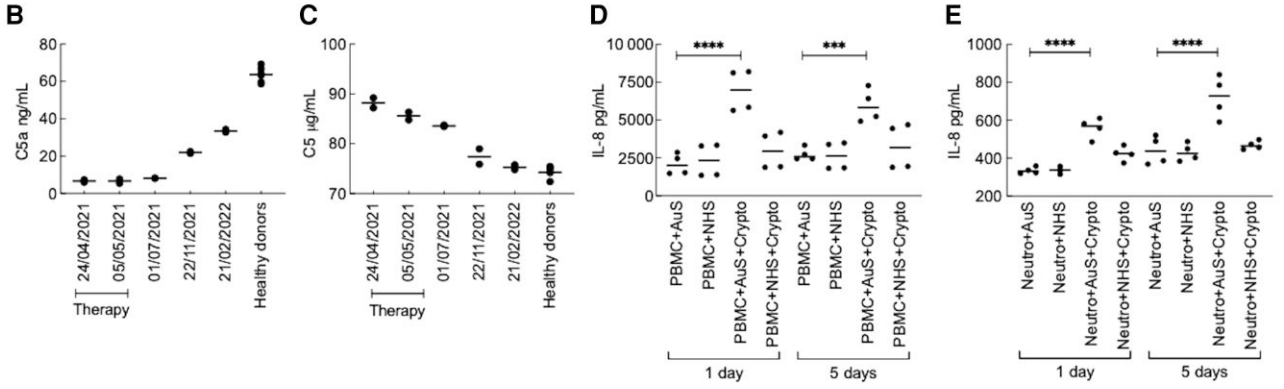

Figure 1. *A*, Complement assay results. *B* and *C*, C5a and C5 levels in the serum of a patient who underwent eculizumab therapy and healthy donors (n = 4). *D* and *E*, Interleukin 8 released by peripheral blood mononuclear cells and neutrophils, respectively, isolated from patient blood samples (collected twice, 18 November 2021 and 21 February 2022), stimulated with *Cryptococcus neoformans* for 1–5 days in RPMI with autologous serum (AuS) or normal human serum; AuS obtained from patient's blood collected on 18 November 2021 was used for the assay, with technical replication. ***$P < .001$; ****$P < .0001$. Abbreviations: AuS, autologous serum; CH50, 50% hemolytic complement activity assay; Crypto, *Cryptococcus neoformans*; FH, factor H; FI, factor I; IL, interleukin; ND, not determined; Neutro, neutrophils; NHS, normal human serum; PBMC, peripheral blood mononuclear cells.

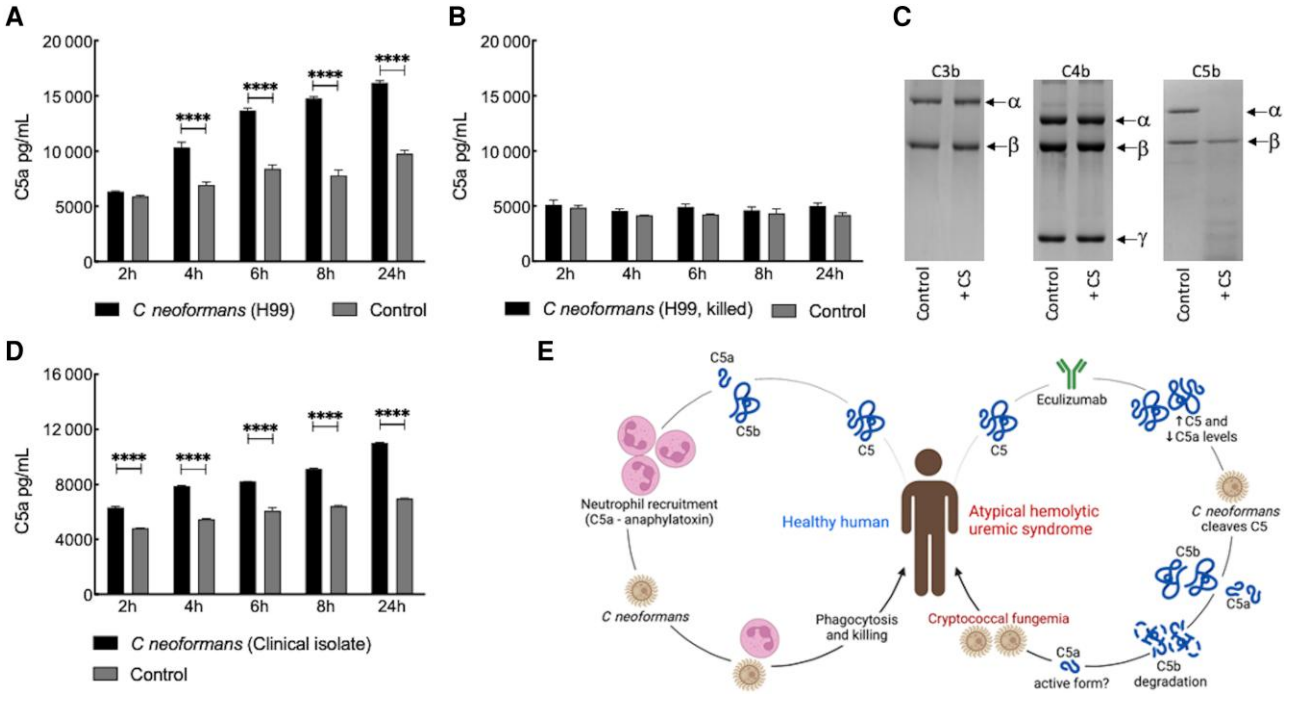

Figure 2. *A*, C5a generated from human C5 treated with *Cryptococcus neoformans* (H99 strain) at specified interaction times. *B*, Heat-killed *C neoformans* (H99) failed to release C5a from C5. *C*, Lytic activity of the culture supernatant (CS) of *C neoformans* (H99) incubated in collagen medium for 24 hours, against different complement proteins. *D*, C5a generating activity of *C neoformans* isolated from the patient. *E*, Postulated mechanism of *C neoformans* infection following eculizumab treatment. ****$P < .0001$.

the degradation of α-chain of C5b (Figure 2*C*). Together, our data suggested that *C neoformans* can noncanonically cleave C5 generating C5a, and further inactivates the other cleavage product C5b. C5a-generating activity was also observed with the *C neoformans* isolated from the patient (Figure 2*D*).

DISCUSSION

Disseminated cryptococcosis occurs in patients with a profound cellular immunodeficiency. Among HIV-negative patients, an increased risk is recognized in patients treated by steroid therapy and/or cellular immunosuppressive agents acting on TNF-α, IL-12, and IFN-γ but not humoral immune defense. Here, we report fungemia due to *C neoformans* following eculizumab therapy.

In an animal model, C5, specifically its cleavage product C5a, has been demonstrated to play a crucial role against circulating *C neoformans* by activating neutrophils, facilitating fungal phagocytosis, and killing [6]. On the other hand, C5a overproduction has been shown to cause immune paralysis and alter neutrophil function [13]. Thus, there is a plausible association with either low or high levels of C5a with risk for cryptococcal fungemia. Our experimental data suggest the pathogenic link between eculizumab therapy and the subsequent occurrence of cryptococcal fungemia. A potential mechanism linking eculizumab with risk of cryptococcal fungemia includes (*i*) a low level of C5a, thereby an attenuated cellular immune defense could be a risk factor for *C neoformans* fungemia; (*ii*) *C neoformans* can cleave C5 noncanonically; (*iii*) eculizumab treatment could divert accumulated C5 for degradation by *C neoformans*; (*iv*) *C neoformans* is endowed with protease(s) that are capable of degrading C5b, a membrane attack complex initiator; and (*v*) noncanonical and excess production of C5a by *C neoformans* may be responsible for amplified IL-8 expression [14] (Figure 2*E*), immune paralysis, and neutrophil dysfunction, thus further favoring *C neoformans* fungemia.

Experimental studies help to better understand the pathogenic cascade. It is demonstrated that the encapsulated *C neoformans* suppresses C5a receptor (C5aR) expression on polymorphonuclear neutrophils [15]. While C5a signaling through its receptor provides survival signal to naive CD4 T cells [16], a low CD4 T-cell count observed in the patient following eculizumab therapy could be related to a lower expression of C5aR. Furthermore, it will be interesting to dissect the activity potential of noncanonically generated C5a upon cleavage of C5 by *C neoformans*. Moreover, although the significance of IL-8 and cryptococcal infection could not be connected precisely in our study, KC(keratinocyte chemoattractant = IL-8) level in the mouse brain with disseminated cryptococcosis is significantly higher [17], and HIV-negative patients with cryptococcal meningitis show elevated IL-8 levels in their CSF [18], suggesting a link between IL-8 and cryptococcosis.

In humans, eculizumab is known to mostly predispose to *Neisseria meningitidis* sepsis but also to other encapsulated bacterial infections [3]. *Cryptococcus neoformans* also being encapsulated, there should be a common pathogenic pathway among these organisms. A recent report showed the development of candidemia and mucormycosis in a patient following eculizumab treatment, suggesting that there could be a fungal-related pathogenic pathway [10]. Although rare, based on our experiments and other reported case studies, disseminated fungal diseases including cryptococcosis should now be added to the list of infections complicating eculizumab therapy.

Notes

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Potential conflicts of interest. For all the authors, there is no conflict to declare.

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