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Secretion of a fungal protease represents a complement evasion mechanism in cerebral aspergillosis

Günter Rambach[a], David Dum[a], Iradj Mohsenipour[b], Magdalena Hagleitner[a], Reinhard Würzner[a], Cornelia Lass-Flörl[a], Cornelia Speth[a,*]

[a] Department of Hygiene, Microbiology and Social Medicine, Innsbruck Medical University, Fritz-Pregl-Straße 3, A-6020 Innsbruck, Austria
[b] Department of Neurosurgery, Innsbruck Medical University, Innsbruck, Austria

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ABSTRACT

Complement represents a central immune weapon in the brain, but the high lethality of cerebral aspergillosis indicates a low efficacy of the antifungal complement attack. Studies with cerebrospinal fluid (CSF) samples derived from a patient with cerebral aspergillosis showed a degradation of complement proteins, implying that *Aspergillus* might produce proteases to evade their antimicrobial potency. Further investigations of this hypothesis showed that *Aspergillus*, when cultured in CSF to simulate growth conditions in the brain, secreted a protease that can cleave various complement proteins. *Aspergillus fumigatus*, the most frequent cause of cerebral aspergillosis, destroyed complement activity more efficiently than other *Aspergillus* species. The degradation of complement in CSF resulted in a drastic reduction of the capacity to opsonize fungal hyphae. Furthermore, the *Aspergillus*-derived protease could diminish the amount of complement receptor CR3, a surface molecule to mediate eradication of opsonized pathogens, on granulocytes and microglia. The lack of these prerequisites caused a significant decrease in phagocytosis of primary microglia. Additional studies implied that the complement-degrading activity shares many characteristics with the previously described alkaline protease Alp1.

To improve the current therapy for cerebral aspergillosis, we tried to regain the antifungal effects of complement by repressing the secretion of this degrading activity. Supplementation of CSF with nitrogen sources rescued the complement proteins and abolished any cleavage. Glutamine or arginine are of special interest for this purpose since they represent endogenous substances in the CNS and might be included in a future supportive therapy to reduce the high lethality of cerebral aspergillosis.

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1. Introduction

While inhalation of conidia of the ubiquitous mould *Aspergillus* is a daily event without major risk for immunocompetent individuals, this contact may be the source of invasive aspergillosis in persons with impaired immune system. If the fungus succeeds to penetrate into the central nervous system (CNS), the subsequent cerebral aspergillosis is associated with an extremely high lethality of >90%, even under high-dosage antimycotic therapy (Ruhnke et al., 2007).

The reasons for the ineffective antifungal immune defense in the CNS are poorly understood. One important parameter is supposed to be the blood–brain barrier that restricts the access of peripheral immune elements to a large extent. This directs the attention to the local immune armamentarium in the CNS like complement, microglial cells and astrocytes. While microglia and astrocytes need to undergo an activation process before being able to fulfill their immune functions, the complement system can be activated within seconds and comprises a battery of potent antimicrobial reactions. Complement, composed of a set of soluble and membrane-bound proteins, is organized as a cascade of consecutive activation steps that are triggered by contact of its pattern recognition molecules with pathogen structures (Rambach et al., 2008b; Speth et al., 2008a). The diverse antimicrobial activities of the complement system that support elimination of pathogens include lysis, opsonization as preparation for the attachment of phagocytes with subsequent ingestion and destruction, chemotactic attraction of immune cells, stimulation of antibody production and T-cell response, modulation of cytokine production and clearance of immune complexes and cell debris. Complement receptors on phagocytes that mediate ingestion of opsonized pathogens complete this arsenal (Speth et al., 2008b; Rambach et al., 2008b).

To examine why complement is unable to clear the fungus from the brain, we studied putative complement evasion mechanisms. Generally, most pathogenic microorganisms have developed a range of strategies to undermine this powerful immune barrier

* Corresponding author. Tel.: +43 512 9003 70705; fax: +43 512 9003 73700.
E-mail address: cornelia.speth@i-med.ac.at (C. Speth).

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and thus to enable the establishment of the infection. The two major principles for complement evasion are avoidance of recognition and avoidance of eradication (Würzner, 1999; Zipfel et al., 2007).

Some facts about complement evasion are already described for *Aspergillus* but are likely to be incomplete. To interfere with complement recognition, putative binding sites on the fungal surface are masked by deposition of pigments. The subsequently moderate binding of complement factor C3 prevents an efficient ingestion by human neutrophils (Tsai et al., 1997, 1998). A strategy of *Aspergillus fumigatus* for avoidance of eradication is the acquisition of complement inhibitor proteins (factor H, FHL-1, FHR-1, C4bp) from the host. Originally designed to protect the body's cells against attack of its own complement system, *A. fumigatus* captures these negative regulators on its surface (Vogl et al., 2008). Furthermore, *A. fumigatus* releases an inhibitor of complement activation that interferes with opsonization, complement-dependent phagocytosis and eradication (Washburn et al., 1986, 1990; Rambach et al., 2008a).

Further studies indicate the presence of another mechanism to avoid eradication by complement attack. Sturtevant (1992; reviewed in Tomee and Kauffman, 2000) revealed the presence of a proteolytic enzyme on the surface of *A. fumigatus* conidia that is able to degrade complement factor C3. Until now nothing is known on the identity of C3-degrading protease(s), nor is it established whether this complement-degrading activity is synthesized when the fungus proliferates in the CNS.

We hypothesized that beside this surface-bound enzyme *Aspergillus* might also produce a protease that is secreted to enable an extensive complement destruction in the CNS compartment and thus to guarantee a broad protection against the antimicrobial capacity of complement. To verify this hypothesis and to analyze whether complement degradation represents a pathogenic mechanism in cerebral aspergillosis, we cultivated *Aspergillus* in CSF and investigated cleavage of complement factors. We could show that the fungus secretes a protease into the CSF that is able to degrade complement proteins and cellular complement receptors in a time-dependent manner. *A. fumigatus*, the by far most frequent causative species of cerebral aspergillosis, owns a higher capacity to destroy complement than other *Aspergillus* species. This complement degradation abolishes the ability of CSF to opsonize fungal hyphae. The relevant proteolytic enzyme is supposed to be a serine-type protease and shares central characteristics with Alp1, an alkaline protease of *A. fumigatus* (Monod et al., 2002); mutants lacking the alp1 gene lack any capacity to degrade complement. The secretion of the enzyme could be repressed by supplementation of the CSF with nitrogen compounds such as arginine and glutamine; that finding might be useful in the future as a supportive therapy in cerebral aspergillosis.

2. Materials and methods

2.1. Aspergillus isolates and culture

A. fumigatus isolates 14 and 15 as well as *A. flavus* isolate 31 and *A. terreus* isolate 57 were obtained from brain samples derived from hospitalized patients with cerebral aspergillosis. The underlying diseases of the patients were acute myeloid leukemia, acute chronic leukemia, and severe aplastic anemia, respectively (Speth et al., 2000). The *A. fumigatus* mutant strain lacking Alp1 was generously given to us by Dr. Elaine Bignell and Dr. David Holden from the Department of Microbiology at the Imperial College London.

Long-term storage of conidia was executed at −80 °C in phosphate buffered saline (PBS) supplemented with 20% glycerol. Experiments were performed with freshly harvested conidia: fungi were grown for at least 5 days on Sabouraud (BD Diagnostic Systems, Franklin Lakes, USA) agar plates at 28 °C until sporulation was clearly visible; conidia were swept off from sporulating colonies with PBS containing 0.05% Tween-20 (Sigma, St. Louis, USA) and kept at 4 °C.

A pool of CSF was obtained from 15 individuals who were investigated for neurological non-inflammatory diseases; aliquots were stored at −80 °C. CSF samples with traces of bleeding or elevated albumin levels were excluded. *Aspergillus* conidia were allowed to germinate overnight in fluid Sabouraud medium (BD Diagnostic Systems) at 37 °C, washed in PBS and then transferred into CSF. The fungal supernatants were harvested at different time points and either used freshly or kept at −80 °C for further disposal. For some experiments CSF was supplemented with glutamine (Sigma), arginine (Sigma), ammonium chloride (Merck, Darmstadt, Germany), ammonium sulfate (Scharlau, Barcelona, Spain), sodium nitrate (Scharlau), saccharose, glucose, galactose (all Sigma), sodium phosphate, potassium phosphate, sodium sulfate or magnesium sulfate (all Merck). Other experiments were performed by adding purified C3 protein (Quidel, San Diego CA, USA) to CSF or to fungal CSF supernatants followed by incubation for 1 h at 37 °C. Protease inhibitor experiments were performed with phenylmethylsulfonyl fluoride (PMSF; Sigma), chymostatin (Sigma), pepstatin (Sigma) or EDTA (Merck), respectively, which were added to the samples prior to C3. Proteolysis of complement proteins was examined by Western blot analysis.

2.2. Western blot analysis

CSF supernatants wherein *Aspergillus* was grown for different time periods were subject to electrophoresis on 9.5% SDS-polyacrylamide gels (SDS-PAGE) under reducing conditions and were subsequently electroblotted onto nitrocellulose. Before probing, blots were blocked in PBS supplemented with 5% skim milk for at least 1 h. For the Western blot analysis a monoclonal or a polyclonal anti-C3 antibody (Santa Cruz, Santa Cruz CA, USA) were used as primary antibodies, as well as polyclonal antibodies against C5 and C1q (Dako, Glostrup, Denmark) and monoclonal antibodies against MBL (Bioporto, Gentofte, Denmark) and factor B (Quidel), followed by a horseradish peroxidase-coupled secondary antibody (Dako). The subsequent detection of the bands was performed by chemoluminescence using LumiGLO Reagent (Cell Signaling Technology, Danvers, USA) and a highly sensitive film (GE Healthcare, Uppsala, Sweden).

2.3. Deposition of complement on fungal germination tubes

The CSF pool was obtained as described above. For opsonization experiments, 5×10^4 conidia were cultured in Sabouraud medium on glass microscope slides at 28 °C until formation of germination hyphae was visible. The adherent fungi were washed with PBS and fixed with acetone and methanol at −20 °C. Fixed fungi were incubated either in CSF or in supernatant of *A. fumigatus* grown in CSF for 4 days. Deposition of the complement factor C3 was detected by standard indirect immunofluorescence procedure after 1 h of incubation. Briefly, the slides were washed with PBS to remove serum or CSF, followed by blocking of unspecific binding with PBS/1% bovine serum albumin (BSA) (Sigma). The specific primary antibody (polyclonal α-C3d; Dako) was added for 1 h at 37 °C. After extensive washing, the fluorescence-labeled secondary antibody (goat-α-rabbit Ig, Alexa 488-labeled; Molecular Probes, Carlsbad, USA) was incubated for 30 min and visualized in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany).

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