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Immune evasion by acquisition of complement inhibitors: The mould *Aspergillus* binds both factor H and C4b binding protein

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Abstact

Pathogenic fungi represent a major threat particularly to immunocompromised hosts, leading to severe, and often lethal, systemic opportunistic infections. Although the impaired immune status of the host is clearly the most important factor leading to disease, virulence factors of the fungus also play a role. Factor H (FH) and its splice product FHL-1 represent the major fluid phase inhibitors of the alternative pathway of complement, whereas C4b-binding protein (C4bp) is the main fluid phase inhibitor of the classical and lectin pathways. Both proteins can bind to the surface of various human pathogens conveying resistance to complement destruction and thus contribute to their pathogenic potential. We have recently shown that *Candida albicans* evades complement by binding both Factor H and C4bp.

Here we show that moulds such as *Aspergillus* spp. bind Factor H, the splicing variant FHL-1 and also C4bp. Immunofluorescence and flow cytometry studies show that the binding of Factor H and C4bp to *Aspergillus* spp. appears to be even stronger than to *Candida* spp. and that different, albeit possibly nearby, binding moieties mediate this surface attachment.
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1. Introduction

In the century of extensive iatrogenic immunosuppression (transplantation, tumour chemotherapy), *Aspergillus* spp. represents a major cause of severe and often lethal, systemic opportunistic fungal infections in immunocompromised hosts.

Invasive aspergillosis is the major infectious cause of death in leukaemia and stem cell transplantation; with *Aspergillus fumigatus* ranked first and *Aspergillus terreus* ranked third according to pathogenicity (Lass-Flörl et al., 2000). *A. terreus* is responsible for 80–100% of deaths caused by invasive aspergillosis, higher than for any of the other 20 pathogenic *Aspergillus* species. Furthermore, *A. terreus* is completely resistant to the powerful antimycotic agent amphotericin B (Johnson et al., 2000). As other supportive care has improved and most bacterial infections can be successfully treated, the importance of aspergillosis has increased, as it is now a major and direct or contributory cause of death in immunocompromised hosts.

Most pathogens invading the human body are attacked by the host immune system directly following entry and usually during further stages of infection. Host defence against fungi depends on phagocytosis, where complement plays a supportive role (Speth et al., 2004). Polymorphonuclear leukocytes (PMN) require complement for maximal chemotaxis, phagocytosis and fungicidal activity. Deposition of C3b on the surface of many invasive pathogens is essential for phagocytic host defence and complement mediated cell lysis (Walport, 2001a,b).

However, several pathogenic micro-organisms have developed specific strategies, including both biochemical or biophysical measures to resist C3b deposition, opsonophago-

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cytosis or complement-mediated cytolytic damage, in order to evade complement and other human immune defence mechanisms. These measures increase the likelihood of micro-organism survival in a hostile environment (Würzner, 1999). The adsorption of host-derived fluid phase complement inhibitors, such as Factor H (FH), factor-H-like protein 1 (FHL-1) or C4b-binding protein (C4bp) inhibits complement activation and has been reported for several micro-organisms (Kraiczy and Würzner, 2006; Würzner and Zipfel, 2004). Employment of these major inhibitors of the alternative and the classical C3 convertase by pathogens results in down-regulation or termination of complement activation (Rooijakkers and Strijp, 2007).

Factor H, FHL-1 and C4bp, similar to other regulators of complement activation (RCA) proteins, are built soley from complement control protein (CCP) modules, also termed short consensus repeats (SCRs). The alternative pathway inhibitor FH consists of 20 SCRs. FHL-1 is composed of 7 SCRs, which are identical to the N-terminal SCRs of Factor H, however with an additional unique C-terminal extension of four amino acids (Zipfel and Skerka, 1999). C4bp, the major inhibitor of the classical and lectin pathways, is the only circulating complement inhibitor with a polymeric structure, the molecule being composed of 6–8 identical α-chains and a single unique β-chain, the α- and β-chains being composed of eight and three short consensus repeats domains, respectively (Blom et al., 2004).

Recently, binding and acquisition of FH, FHL-1 and C4bp was shown for *C. albicans* (Meri et al., 2002,2004). Importantly, these proteins maintain their complement regulatory functions in their bound configuration, resulting in down-regulation or termination of the complement cascade (Meri et al., 2002,2004).

The present study evaluates complement evasion by moulds such as *A. fumigatus* and *A. terreus*, both of which, besides *C. albicans*, are major causes of severe, systemic fungal infections in immunocompromised hosts.

2. Methods

2.1. Strains and growth conditions

Culture collection strains of *C. albicans* (SC5314 and CBS 5982), *C. dubliniensis* (CD38, D. Coleman, Dublin, Ireland, (Sullivan and Coleman, 1998; Gilfillan et al., 1998)) or Saccharomyces cerevisiae (Deutsche Stammsammlung für Mikroorganismen, Braunschweig, Germany (DSM) 70451) were grown on Sabouraud dextrose agar (1% peptone (Becton Dickinson, Heidelberg, Germany), 4% glucose (AppliChem, Neudorf, Austria)) and transferred to RPMI medium (GIBCO-Invitrogen, Vienna, Austria) for 16 h at 30 °C (predominantly yeasts present) or 37 °C (mostly hyphae present, only for *Candida*) for immunofluorescence (IF).

For fluorescence-activated cell analyses (FACS), colonies were washed off with FACS buffer from Sabouraud dextrose agar culture plates, previously grown at 37 °C for two days, fitrated through a 40 μm Nylon cell strainer (Becton Dickinson) and stored at 4 °C.

Culture collection strains of *A. fumigatus*, *A. terreus*, *A. niger* and *A. nidulans* (American Type Culture Collection, Rockville, MD (ATCC) 204305, DSM 826, ATCC 9142 and ATCC 38163, respectively) were used for all experiments. Cultures were obtained from Sabouraud dextrose agar, either washed off with phosphate-buffered saline (for IF) or washed off with FACS buffer.

2.2. Plasma, antibodies, and proteins

Plasma was obtained from healthy human donors; EDTA was added at a concentration of 10 mM.

Factor H was obtained from Merck (Darmstadt, Germany). FHL-1, FH SCRs 8–20 (Zipfel et al., 1994) and both the complete form of C4bp and a construct without β-chain and the attached protein S were generated or purified as described (Blom et al., 2001,2004).

A polyclonal rabbit antiserum raised against FH SCRs 1–4 antiserum for the detection of the N-terminal SCRs of Factor H and FHL-1 and a polyclonal rabbit IgG for the detection of the C-terminal SCRs 19–20 of Factor H (Zipfel et al., 1994) were used for detection of Factor H constructs in both IF and FACS. A polyclonal goat antiserum raised against human Factor H antiserum was obtained from Quidel (San Diego, CA).

The monoclonal mouse antibody 104 recognizing SCR1 of the C4bp α-chain (Berggard et al., 2001a) and a polyclonal rabbit antibody (ab9008) (Kask et al., 2002) were used for detection of C4bp.

Rabbit IgG, goat IgG and mouse IgG (obtained from the Department of Immunology, University of Göttingen) were used as control.

FITC-conjugated goat anti-mouse, swine anti-rabbit and rabbit anti-goat antisera were purchased from Dako (Glostrup, Denmark).

2.3. Indirect immunofluorescence (IF)

Fungal hyphae were incubated with human EDTA-plasma (50%), C4bp or C4bp construct without ß-chain (both 20 μg/ml) for 4 h at 4 °C, followed by three washing steps using ice-cold PBS. Cells were incubated on slides coated with poly-L-lysine (Sigma–Aldrich, St. Louis, MO) for 30 min. After fixation (3 min in acetone/methanol) and blocking (15 min, 1% PBS-FCS) at room temperature, primary antibodies were incubated for 1 h (goat anti-Factor H 1:50; rabbit anti-FH SCRs 1–4, rabbit anti-FH SCRs 19–20 or rabbit anti-C4bp, all 20 μg/ml; controls goat IgG or rabbit IgG, both 20 μg/ml). Detection was performed via secondary FITC-labelled antibodies at a 1:40 dilution. Cells were counterstained with EvansBlue® (Sigma–Aldrich) and covered for immunofluorescence with Mowiol (Sigma–Aldrich). Analyses were done immediately thereafter.

2.4. Flow cytometry

Conidia (from *Aspergillus* spp.) or yeast cells (from *Candida* spp.) were incubated at 2×10^6 ml^{-1} (final concentration) with human EDTA-plasma (50%), Factor H, FHL-1, FH SCRs 8–20 or C4bp or the C4bp construct lacking the ß-chain (all 20 μg/ml) for 2 h at 4 °C. After two washing steps with ice-cold

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