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The control of the complement lectin pathway activation revisited: Both C1-inhibitor and antithrombin are likely physiological inhibitors, while α_2-macroglobulin is not

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ABSTRACT

The lectin pathway of complement is an important effector arm of innate immunity. It forms a first line of defense against invading pathogens and dangerously altered self structures. Pattern recognition molecules (mannose-binding lectin (MBL), ficolins) bind to the dangerous particles, which is followed by activation of MBL-associated serine proteases, MASP-1 and MASP-2, resulting in the initiation of the complement cascade. The activation of the lectin pathway is strictly controlled by natural inhibitors, since uncontrolled activation can lead to serious self-tissue damage. Recently we have shown that inhibition of either MASP-1 or MASP-2 by *in vitro* evolved specific inhibitors completely blocks the lectin pathway in human serum. In this study, we examined the inhibitory action of C1-inhibitor (C1-inh), antithrombin (AT) and α_2-macroglobulin (α_2M) on MASP-1 and MASP-2, and studied the inhibition of the lectin pathway in normal human serum in the presence and absence of heparin using C3 and C4 deposition assays. We measured the association rate constants for the serpin/protease reactions. We found that in the presence of heparin both C1-inh and AT are equally efficient inhibitors of the lectin pathway. Although α_2M formed complex with MASP-1 in fluid phase, it could not abolish lectin pathway activation on activator surfaces.

1. Introduction

The complement system forms an important effector arm of the innate immunity. It can be activated by invading pathogens (PAMPs: pathogen associated molecular patterns) or by altered self structures (DAMPs: damage associated molecular patterns). Complement activation results in elimination of the target structures and initiates inflammatory reactions (Ricklin et al., 2010). The complement system can be activated via three different routes: the classical, the lectin and the alternative pathways. Although the lectin pathway was discovered 25 years ago (Ikeda et al., 1987), new components have been identified recently and there are many open questions concerning the activation and the regulation of the pathway (Yongqing et al., 2012). According to our present knowledge there are five pattern recognition molecules that can trigger the activation of the lectin pathway: mannan-binding lectin (MBL) (Dommett et al., 2006), three ficolins (H-, L- and M-ficolin) (Endo et al., 2011) and collectin 11 (CL-11 or CL-K1) (Hansen et al., 2010). The pattern recognition molecules form complexes with the multidomain MBL-associated serine proteases (MASPs), namely MASP-1, MASP-2 and MASP-3 (Gál et al., 2009). Two non-catalytic alternative splice products of the *MASP1* and *MASP2* genes, MAp44 (MAP-1) (Degn et al., 2009; Skjoedt et al., 2010) and MAp19 (sMAP) (Stover et al., 1999; Takahashi et al., 1999), respectively, were also detected in the initiation complexes. The serine proteases are present as zymogens in the initiation complexes and become activated only after the recognition subunits bind to the activator structures (e.g. carbohydrate arrays on pathogen surface). For a long time MASP-2 has been regarded as the key protease of the lectin pathway since it can autoactivate and cleave C4 and C2 to generate the C3 convertase complex (C4bC2a) (Thiel et al., 1997; Vorup-Jensen et al., 2000; Chen and Wallis, 2004). Contrary to this picture we have shown recently that MASP-1, the most abundant MBL-associated protease, controls the lectin pathway activation in normal human serum (Héja et al., 2012b). The first step of lectin pathway activation is the autoactivation of MASP-1 which is followed by the MASP-1 mediated cleavage of MASP-2. MASP-1 is the exclusive activator of MASP-2 and inhibition of the proteolytic

Abbreviations: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; C1-inh, C1-inhibitor; AT, antithrombin; α_2M, α_2-macroglobulin; k_a, association rate constant; PAMP, pathogen associated molecular pattern; DAMP, damage associated molecular pattern; CCP, complement control protein; SP, serine protease; rMASP, recombinant MASP CCP1-CCP2-SP.

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activity of MASP-1 prevents activation of MASP-2. Our conclusions were reinforced by using serum from a patient with MASP-1 deficiency (Degn et al., 2012). Moreover, it was also suggested that MASP-1 is the activator of MASP-3 (Iwaki et al., 2011; Degn et al., 2012). Now it is clear that MASP-1 is the key initiator protease of the lectin pathway and inhibition of MASP-1 prevents the activation of the entire pathway.

In this study we examined the inhibitory effect of several serum inhibitors on the lectin pathway in the light of the most recent discoveries concerning the mechanism of activation. We showed that AT in the presence of heparin is nearly as efficient inhibitor of the lectin pathway activation as C1-inh. Surprisingly, however, α_2M, which forms complex with the catalytic fragment of MASP-1 in the fluid phase, could not attenuate C4 and C3 deposition from normal human serum on mannan coated plates.

2. Materials and methods

2.1. Reagents

AT was prepared from pooled, fresh-frozen human plasma as described by Dobó et al. (2009) with some modifications. In brief, the plasma was filtered, loaded onto a Heparin Sepharose 6 Fast Flow column (GE Healthcare), washed with 50 mM Tris, 0.1 mM EDTA, 200 mM NaCl, pH 7.4 (equilibration buffer), then AT was eluted using a 20-column-volume linear salt gradient (0.2–2 M NaCl). The AT containing fractions were purified by anion exchange chromatography (on Source 30Q from GE Healthcare), concentrated, and stored at −20 °C. Concentration of AT was calculated based on the molecular mass of 58 kDa, and the extinction coefficient of 0.65 ml mg^{-1} cm^{-1} (Olson et al., 1992).

Lyophilized C1-inh containing buffer (Berinert-P from CSL Behring) was reconstituted with sterile water and stored at −20 °C in aliquots. Concentration of C1-inh was calculated based on the molecular mass of 71 kDa, and the extinction coefficient of 0.382 ml mg^{-1} cm^{-1} (Dobó et al., 2011).

The catalytic fragments of human MASP-1 and MASP-2 containing the two complement control protein (CCP1 and CCP2) and the serine protease (SP) domains (rMASP-1, rMASP-2) were expressed in *Escherichia coli* and prepared according to Dobó et al. (2008) and Ambrus et al. (2003) in the absence of inhibitors.

α_2M was purified from pooled, fresh-frozen human plasma as previously described (Sottrup-Jensen et al., 1980) with several modifications. 1 mM Pefabloc SC, 100 μM NPGB, and 10 mM EDTA was added to the plasma, then it was precipitated with PEG-3350 (4–12 w/v%). The precipitate was dissolved in 50 mM Na-phosphate, 250 mM NaCl, 10 mM imidazole, 0.02% NaN_3, pH 7.4 buffer, and the sample was loaded onto a Ni-NTA column (Qiagen). After washing with 50 mM Na-phosphate, 250 mM NaCl, 40 mM imidazole, 0.02% NaN_3, pH 7.4 (wash buffer), α_2M was eluted with 50 mM Na-phosphate, 250 mM NaCl, 250 mM imidazole, 0.02% NaN_3, pH 7.4 (elution buffer). 0.5 mM Pefabloc SC was added to the α_2M containing fractions, which were dialyzed overnight at 4 °C against 20 mM TRIS, 0.1 mM EDTA, pH 7.5. After loading to an anion exchange column (Q Sepharose HP from GE Healthcare) α_2M was eluted using a 100–400 mM NaCl gradient. Finally the concentrated sample was gel-filtrated on a preparative Superdex 200 column (GE Healthcare) in 20 mM Tris, 0.1 mM EDTA, 150 mM NaCl, pH 7.5. α_2M containing fractions were concentrated and stored at −20 °C in aliquots. The concentration of α_2M was calculated based the molecular mass of 720 kDa (tetrameric form), and the extinction coefficient of 0.9 ml mg^{-1} cm^{-1} (Sottrup-Jensen et al., 1980).

Heparin and BSA were from Sigma (H0777, and A6003). Acetylated BSA was prepared by incubation of 10 mg/ml BSA in 0.5 M HEPES, pH 7.4 with acetic anhydride in about threefold molar excess to amino groups at 20 °C (Héja et al., 2012b).

2.2. Inhibition assay

The activity and inhibition of rMASP2 was measured in 50 mM HEPES, 140 mM NaCl, 0.1 mM EDTA, 0.1% PEG-3350, pH7.4 buffer, using the thioester substrate Z-Gly-Arg-S-Bzl (MP Biomedicals) at 40 μM, and the thiol reagent 4,4′-dithiodipyridine (DTDP) at 80 μM concentration. AT was used in the assay at 0, 1, 4, or 7 μM and C1-inh was used at 0, 50 or 200 nM concentration. The final rMASP-2 concentration was 100–500 pM, and the concentration of heparin was in the range of 0–500 μg/ml. The Michaelis-Menten constant (K_M) for rMASP-2 and Z-Gly-Arg-S-Bzl was 9.3 ± 2.5 μM. The second order association constants (k_a) were determined under pseudo-first-order conditions. Data were fitted by nonlinear regression using the $A_t = a - b \times \exp(-k_{obs} \times t) + c \times t$ equation, where A_t is the absorbance; a, b, and c are fitting parameters; and k_{obs} is the observed association rate constant (Dobó and Gettins, 2004). The second order rate constants (k_a) were calculated (Schechter and Plotnick, 2004) as $k_a = (k_{obs}/[I]_0) \times (1 + [S]/K_M)$, where $[I]_0$ is the serpin concentration, [S] is the substrate concentration, and K_M is the Michaelis-Menten constant for the protease and substrate.

2.3. C3 deposition assay from human serum

C3 deposition was measured as described previously (Kocsis et al., 2010) with some modifications. Shortly, Greiner high-binding microtiter plates were coated with 10 μg/ml mannan, or 50 μg/ml acetylated BSA in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH9.6 (coating buffer). After overnight incubation at 4 °C, wells were blocked with 1% BSA in TBS buffer (20 mM TRIS, 150 mM NaCl, pH7.4) for 1 h at 37 °C, then washed with TBS containing 0.05% Tween-20 and 5 mM $CaCl_2$ (wash buffer). Serum was diluted 100-fold in 10 mM HEPES, 145 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween-20, pH7.4 and was incubated in microcentrifuge tubes with different concentrations of added AT, C1-inh, α_2M and heparin for 30 min at room temperature. Then the samples were added to the microtiter plate and incubated for 30 min at 37 °C. The plate was washed three times with wash buffer and the deposited C3 was detected by polyclonal rabbit anti-human C3c (A0062; DakoCytomation, Glostrup, Denmark) and anti-rabbit-HRP (A1949, Sigma) antibodies (Kocsis et al., 2010).

2.4. C4 deposition assay

C4 deposition was measured analogously as described for the C3 deposition assay. C4 was detected with polyclonal rabbit anti-human C4c (Q0369; DakoCytomation, Glostrup, Denmark), and anti-rabbit-HRP (A1949, Sigma) antibodies.

2.5. Deposition of C4 by preactivated MASP-2

The C4 deposition by preactivated MASP-2 was measured similarly to Petersen et al. (2001). After blocking the mannan coated wells with 1% BSA in 20 mM HEPES, 140 mM NaCl, 5 mM EDTA, 0.1% Tween-20, pH 7.4 (blocking buffer), the wells were incubated with 50-fold diluted serum in 40 mM HEPES, 2 M NaCl, 10 mM $CaCl_2$, pH 7.4 (high salt serum dilution buffer) for 1 h at 4 °C. High salt conditions allow binding of MBL–MASP complexes to mannan, but prevent subsequent complement activation. The wells were then washed three times with 20 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 0.1% Tween-20, pH 7.4 ("High salt" buffer) and three times with 20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 0.1% Tween-20, pH 7.4 (normal wash buffer). Purified C4 (700 μg/ml) and different concentrations of C1-inh and AT in the presence or absence of 50 μg/ml heparin were added in normal wash buffer to the wells and incubated for 1 h at 37 °C. After three washes with blocking buffer, deposited