New functional ligands for ficolin-3 among lipopolysaccharides of Hafnia alvei

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Ficolin-1 (M), ficolin-2 (L), ficolin-3 (H) and mannan-binding lectin (MBL) activate the complement and have opsonic activity. The specificity of ficolin-3 is poorly characterized and currently limited to a few ligands only. We present new specific targets for human ficolin-3, identified among lipopolysaccharides (LPSs, endotoxin) of Hafnia alvei. The interaction was restricted to LPSs of four strains: 23, Polish Collection of Microorganisms (PCM) 1200, PCM 1203 and PCM 1205 and limited to their O-specific polysaccharides (O-specific PSs) composed of different numbers of oligosaccharide (OS) repeating units (RUs). Moreover, these LPS/ficolin-3 complexes activated the lectin pathway of complement in a C4b-deposition assay in a calcium- and magnesium-dependent way. A neoglycoconjugate of the O-specific PS fraction of H. alvei 1200 LPS with bovine serum albumin (BSA) was prepared and used as a tool for the determination of ficolin-3 concentration and activity in serum. To confirm a structure of the O-specific PS 1200 selected for the conjugate preparation, structural analysis was performed on a series of O-specific PSs released by the mild acid hydrolysis of the LPS. The isolated O-specific PSs, showing the different length distributions, were devoid of a major part of the core OS region and had Hep-Kdo disaccharide at a reducing end. The neoglycoconjugate was a highly selective tool for the determination of ficolin-3 concentration and activity in serum (lectin pathway activation in the C4b deposition assay) and was not affected by MBL, ficolin-1 and ficolin-2 or natural antibodies.

Keywords: complement / endotoxin / ficolin / Hafnia / lipopolysaccharide

Introduction

Many of the mechanisms of innate immunity depend on fast, flexible and straightforward interactions between soluble or cell-associated pattern-recognition molecules (PRMs) and pathogen-associated molecular patterns (PAMPs)-conservative structures common for the variety of pathogens. Examples of such PRR-PAMP are the collagen-associated lectins, i.e. ficolin-1 (M), ficolin-2 (L), ficolin-3 (H, Hakata antigen) and mannan-binding lectin (MBL), together with their microbial ligands. These molecules are involved in the complement activation, via the lectin pathway, an important element of innate immune response. This unique feature depends on the ability of these lectins to form complexes with MBL-associated serine proteases (MASPs; Matsushita 2007). The C-terminal fibrinogen-like region of ficolins constitutes the target-recognizing domain (MacDonald and Kilpatrick 2007; Matsushita 2007; Endo et al. 2011). Identification of ligands for ficolins is a prerequisite to understand the role of the collagen-related lectins in innate immunity and to design diagnostic tools to measure concentration and the activity of these key complement components in body fluids, e.g. serum.

Ficolin-3 is an oligomer of subunits consisting of three identical polypeptide chains. Despite similar molecular organization, biochemical properties and activities, it differs from other proteins of that family as the amino acid sequence homology between ficolin-3 and ficolin-1 or ficolin-2 is 48% (Matsushita 2007; Thiel 2007; Hummelshoj et al. 2008; Matsushita 2009; Garred et al. 2010; Endo et al. 2011). Ficolin-3 is synthesized in the liver by hepatocytes and bile duct epithelial cells and in lungs by type II alveolar and ciliated bronchial epithelial cells. It is secreted into blood, bile ducts, bronchi and alveoli (Matsushita 2007); thus, it may participate in both systemic and local innate immune response.

The average concentration of ficolin-3 in human sera is 18 µg/mL, the highest among ficolins and significantly exceeding the MBL level (Yae et al. 1991). The importance of serum ficolin-3 was indicated by several reports concerning disease associations with abnormally low or high concentrations. Lower levels of ficolin-3 were observed among patients suffering from systemic lupus erythematosus (Inaba et al. 1990) and sarcoidosis (Svendsen et al. 2008). A reduced ficolin-3
level in hepatic cirrhosis may be a marker of impaired liver function (Fukutomi et al. 1996). Schlapbach et al. demonstrated recently an enhanced risk of febrile neutropenia (especially with bacteremia) in ficolin-3-deficient pediatric cancer patients, treated with chemotherapy (Schlapbach et al. 2009) as well as necrotizing enterocolitis among neonates (Schlapbach et al. 2010). Wang et al. (2007) reported high concentrations of ficolin-3 in the placentas from preeclamptic pregnant women, accompanied by low serum levels.

Considering human ficolins, the specificity of ficolin-3 is rather poorly characterized. Only a few ligands have been identified to date. The first communication concerning targets for ficolin-3 dealt with its ability to agglutinate erythrocytes coated with lipopolysaccharide (LPS, endotoxin, O-antigen) of *Salmonella Typhimurium*, S. Minnesota and *Escherichia coli* O111 (Sugimoto et al. 1998). Tsujimura et al. (2002) identified an exopolysaccharide (EPS) of *Aerococcus viridans* as a ligand for ficolin-3, but its detailed structure has not been elucidated to date. No ligands were identified among numerous strains of streptococci, staphylococci and *E. coli* (Krarup et al. 2005). N-Acetylated BSA (BSA-NAc) was the last ligand identified to date. This artificial ligand was reported to be useful for the determination of ficolin-3-dependent complement activity (Lacroix et al. 2009; Munthe-Fog et al. 2009; Hein et al. 2010) and was used to confirm the first case of ficolin-3 deficiency in a patient suffering from recurrent infections (Munthe-Fog et al. 2009).

Herein we present a part of our continuous studies of structure–biological activity relationships for LPSs, that have led to the identification of several *Hafnia alvei* LPSs as new ligands bound by ficolin-3.

*Hafnia alvei*, a Gram-negative bacterium, is a causative agent of bacteremia and sepsis in humans and animals. It is also associated with respiratory diseases and mixed hospital infections in humans. Sepsis developed due to *H. alvei* infection is also a serious clinical problem in the animal production industry of commercial laying hens, pullets and rainbow trout (Janda et al. 1999). LPSs of *H. alvei* are major surface antigens, composed of three main regions lipid A, core oligosaccharide (OS) and the O-specific PS, consisting of different numbers of OS repeating units (RUs). The biosynthesis and assembly of LPSs is a complex multi-step process comprising the polymerization of the O-specific chain and its subsequent ligation onto the outer core domain of the lipid A–core OS acceptor (Raetz and Whitfield 2002). The O-specific PS is usually characterized by different degrees of polymerization due to the varying number of RUs attached to the core OS. The degree of polymerization is a main reason of heterogeneity observed among LPSs isolated from one particular strain. Preparations of smooth-type LPS usually constitute a mixture of molecules composed of lipid A substituted by core OS and different numbers of RUs as well as lipid A substituted by core OS only. The O-antigens of *H. alvei* are subdivided into 40 O-serotypes (Baturo and Raginskaya 1978; Romanowska 2000). The structures of the O-specific PS from more than 30 serologically different *H. alvei* strains have been elucidated (Romanowska 2000; Katzenellenbogen et al. 2005; Lukasiewicz et al. 2009). In the case of core OS, only four types have been identified to date. Unlike almost all of entero bacterial endotoxins, Kdo-containing motifs were identified in the outer core region of *H. alvei* LPS (Lukasiewicz et al. 2009).

Herein, we present newly identified interactions between ficolin-3 and some of *H. alvei* LPSs. We have identified regions of LPS recognized by this lectin and present these ligands as a potential diagnostic tool for the determination of ficolin-3 concentration and ficolin-3-dependent lectin pathway activity measurements in body fluids.

Results

Interaction of serum ficolin-3 with *H. alvei* LPSs

Forty-five *H. alvei* LPSs were tested in a dot-bl ot assay to find these being recognized by serum-derived ficolin-3 (data not shown). Four LPSs isolated from strains, 23, PCM 1200, PCM 1203, PCM 1205, were reactive and were further analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)/Western blot using human sera as the source of ficolin-3 or ficolin-2 (serum A), MBL (serum B) or ficolin-1 (poled A and B sera). This was done to identify a region within LPSs recognized by ficolin-3. SDS–PAGE combined with silver staining of smooth-type LPS usually gives a characteristic ladder-like multi-band pattern. The fast-migrating fractions originate from lipid A substituted with core OS. The slower-migrating fractions show the length distribution of the polymer built of lipid A–core OS substituted with different numbers of RUs (Figure 1A). The ficolin-3 bound to high-molecular-weight fractions of LPS, which indicated the O-specific PS (with different numbers of RUs) as a region recognized by this lectin (Figure 1A and B). LPS isolated from *H. alvei* PCM 1192 was used as a negative control. In contrast, MBL interacted with low-molecular-weight LPS fractions of 1192, 1200, 1203 and 1205 strains, corresponding to the lipid A-core region (Figure 1A and C). The reactivity of ficolin-2 and ficolin-1 with LPSs tested was negligible (data not shown). Efficient binding of ficolin-3 to the aforementioned LPSs was also confirmed by ELISA, with a detectable (A405 nm > 0.2) reaction for serum dilutions 1:800–1:1600 (Figure 2A).

Complement activation

Ability of LPS 23, 1200, 1203, 1205 to initiate the lectin pathway of the complement activation through a formation of LPS/ficolin-3/MASP-2 complexes and cleavage of C4 was demonstrated by a C4b deposition assay (Figure 2B). LPS isolated from *H. alvei* PCM 1192 was used as a negative control.

Inhibition of ficolin-3 binding to LPS 1200 by ion chelators

LPS 1200 was used as a solid phase in ELISA and the binding of ficolin-3 was measured in the presence or the absence of EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid). The observed interaction between ficolin-3 and LPS *H. alvei* 1200 was calcium- and magnesium-dependent. It was strongly inhibited by both EDTA and EGTA and, to some extent, was reconstituted by Mg²⁺ supplementation (EGTA/Mg²⁺; Figure 2C).
Binding of recombinant ficolin-3 to bacterial cells

Flow cytometry was used to demonstrate the binding of recombinant human (rh) ficolin-3 to cells of *H. alvei* PCM 1200 (Figure 3). This result suggests that ficolin-3 recognizes not only isolated endotoxin, but also LPS as an integral component of the bacterial outer membrane. No interaction was observed in the case of the control strain: *H. alvei* 1192 (data not shown).

The specificity of the ficolin-3 to LPS present on the surface of bacteria was examined by an ELISA inhibition test with the *H. alvei* 1200 cells used as a solid phase and LPS 1200 as the inhibitor (Supplementary data, Figure S2). LPS 1200 showed 50% and 100% inhibition of the ficolin-3 binding at 29 and 1875 ng of LPS, respectively. This suggested that there are no other ficolin-3-binding motifs at the surface of *H. alvei* 1200 bacteria.

Analyses of the O-specific PSs of *H. alvei* 1200 LPS

SDS–PAGE/Western blot analysis of *H. alvei* LPS/ficolin-3 interactions led to the identification of the O-specific chain as a region being recognized by this lectin. In order to define an entire structure of the O-specific PS, especially reducing ends of these PSs, structural analysis were performed on a series of the O-specific PSs isolated from LPS 1200. This information was a prerequisite for further preparation of neoglycoconjugate (coupling chemistry selection) to use as a tool for ficolin-3 concentration and ficolin-3-dependent lectin pathway activity measurements in body fluids.

The O-specific PS of LPS 1200 was chosen for this purpose due to its reactivity with ficolin-3 and the known structure of the RU identical to this present in LPS 1203 and 1205 (Dag et al. 2004). However, in-depth analysis of the O-specific PS fractions released upon the mild acid hydrolysis of LPS 1200 was necessary for the identification of sugar residue(s) present at the reducing end of these O-specific PSs.

LPS of *H. alvei* 1200 was hydrolyzed with the use of 1.5% acetic acid resulting in a cleavage of a ketosidic linkages present in LPS structure, including the linkage between the Kdo residue of the core OS and distal GlcN of lipid A. The fractionation of products on Bio-Gel P-10 yielded five PS fractions (PS1, 38 mg; PS2, ≈1.5 mg; PS3, ≈1.5 mg; PS4, ~1 mg; PS5, ~1.2 mg) and two OS fractions (OS1, 37 mg; OS2, 14 mg). Fraction PS1 was analyzed previously by Dag et al. (2004) and the RU structure of the LPS 1200 O-specific PS was identified. All fractions (PS1, PS2, PS3, PS4, PS5 and OS1) were checked using 1H NMR and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

MS spectra were obtained for fractions PS5, PS4 (Figure 4B and C) and OS1 that showed all structural features of the O-specific PSs isolated from LPS 1200. Interpretation of mass spectra (Table I) indicated that PS5 and PS4 differed only in a degree of polymerization (different numbers of RUs). Major ions present in the mass spectra of PS4 and PS5 corresponded to three and two RUs linked to the Hep-Kdo disaccharide at the reducing end of these PSs. The MALDI-TOF mass spectrum of the PS5 (Figure 4B) showed the major pair of ions at m/z 2452.07 and 2494.08 corresponding to two RUs linked to the Hep-Kdo disaccharide and additionally substituted with one or two *O*-acetyl groups, respectively. As for PS4 (Figure 4C), [M–H]− ions at m/z 3607.66 and 3565.67 corresponded to the structures built of three RUs linked to the Hep-Kdo disaccharide and substituted with three or two *O*-acetyl groups, respectively. The OS1 mass spectrum (data not shown) showed ions attributed to core OSs and additionally ions at m/z 1380.99 and 1338.97 corresponding to one RU substituting the Hep-Kdo disaccharide (Table I).

The analysis indicated the presence of Hep-Kdo disaccharide in the O-specific PSs in fractions PS4, PS5 and OS1. According to the rules of LPS biosynthesis (Raetz and Whitfield 2002), this structural motif is also present in O-specific chains with a higher degree of polymerization—PS3, PS2 and PS1 (built of ≥4 RUs). This indicated a general structure of the O-specific PSs (PS1–PS5) isolated from LPS 1200 (Figure 4A).

NMR analysis was performed for PS5 in order to identify a location of the Hep-Kdo disaccharide. According to MS data, PS5 represents the O-specific PS with the highest degree of polymerization (two RUs) for which NMR analysis of the

Fig. 1. Silver stain SDS–PAGE (A) and Western blotting analysis of the interaction of human serum-derived ficolin-3 (B) and MBL (C) with LPSs of *H. alvei*. Lanes are depicted with the strain number. Normal human serum A and B, 30-fold diluted, was used as a source of ficolin 3 and MBL, respectively.

![Image](https://academic.oup.com/glycob/article-abstract/22/2/269/1987982/1.5?download=true)
The entire molecule including Hep-Kdo disaccharide is still feasible. The one-dimensional $^1$H-NMR and two-dimensional $^1$H, $^{13}$C-HSQC (heteronuclear single quantum coherence)-DEPT (distortionless enhancement by polarization transfer) NMR spectra of the PS5 (Figure 5) contained 12 signals in the anomeric region of the spectra and a Kdo spin system. All identified spin systems are presented in Supplementary data, Table SI. The chemical shift values were compared with the previously published NMR data for the RU of the O-specific PS (PS1) isolated from $H$. alvei $^{1200}$ LPS (Supplementary data, Figure S1; Katzenellenbogen et al. 1992, 1999; Dag et al. 2004). The results indicated a basic structure of the RU, which is in agreement with previously published data (Dag et al. 2004; Figure 5). Since the structure of the RU of LPS 1200 is already known, a full description of the spin systems was limited to newly identified characteristic structural elements of PS5 ($A$, $B$, $C$ and $h$), indicating the reducing and non-reducing ends. The remaining residues were presented in Supplementary data (Tables SI and SII). The sugar residues identified are denoted by upper-case letters (RU1, Kdo and Hep residues) or lower-case letters (RU2) as shown in the structure of the isolated PS5 (Figure 5).

Residue $A$ was identified as a 7,8-disubstituted 3-deoxy-$\alpha$-d-manno-oct-2-ulospyranosonic acid (Kdo) on the basis of characteristic deoxy proton signals at $\delta$ 1.80 and 2.05 ppm (Figure 5, inset spectrum), and a chemical shift of the C-7 ($\delta$ 75.7 ppm) and C-8 ($\delta$ 64.4 ppm) signals (Birnbaum et al. 1987; Vinogradov and Bock 1999).

Residue $B$ with the H-1/C-1 signals at $\delta$ 4.77/99.3 ppm was recognized as the terminal $\alpha$-glycer-o-$\alpha$-d-manno-Hepp residue due to the small vicinal couplings between H-1, H-2 and H-3 and similar chemical shifts as in the monosaccharide $\alpha$-C-2-Hep (Vinogradov and Bock 1999).

Residue $C$ with the H-1/C-1 signals at $\delta$ 4.74/100.4 ppm was assigned as the 3-substituted $\beta$-GlcNAc residue (RU1 constituent) based on the low chemical shift of the C-2 signal.
(δ 56.8 ppm), the relatively high chemical shift of the C-3 signal (δ 82.5) and the large vicinal coupling constants between all ring protons.

Residue h at 4.44/102.8 ppm was recognized as the terminal β-D-Qui4NAc residue (RU2 constituent) based on the characteristic chemical shift of the C-4 signal (δ 56.8 ppm) and the signal for an exocyclic CH₃ group (δ 1.20 and 16.9 ppm).

The heteronuclear multiple-bond correlation (HMBC), nuclear Overhauser effect spectroscopy (NOESY) and rotating-frame Overhauser effect spectroscopy (ROESY) experiments (Supplementary data, Table SII) showed inter-residue connectivities between adjacent sugar residues and thus provided the sequence in PS5 (Figure 5). The linkage between residues C and A was deduced from an indicative cross-peak in the HMBC spectrum between C-1 of residue C...
and H-7 of residue A (Supplementary data, Table SII) and the Kdo substitution pattern. The newly identified element, →3)β-D-GlcNAc-[α-L-2′-Ac]-[1,3]-Kdo (residues C, B and A), indicated the reducing end of the PS5, thus the linkage between the first RU of the O-specific PS and the Hep-Kdo disaccharide. The presence of terminal β-D-ManP4NAc (h) indicated the structure of the biological RU of LPS 1200 (Figures 4A and 5). Additionally, in agreement with studies of Katzenellenbogen et al. (2005), anomeric configuration of β-GlcNAc (residue C) in RU1 is altered from α to β, compared with that of the subsequent RUs of the O-specific PS (e.g. RU2). The herein presented structural data indicated that regardless of the number of RUs, there was always the Hep-Kdo disaccharide at the reducing end as a linking element between the O-specific chain and the core OS of the LPS.

Conjugation of the reduced PSI with BSA

Discovered interaction prompted us to synthesize a glycoconjugate of PSI isolated from LPS 1200 with BSA (PSI-BSA) in order to prepare a tool for ficolin-3 concentration and ficolin-3-dependent lectin pathway activity measurements in body fluids. The conjugation was performed according to Lönngren and Goldstein (1978), through the Kdo residue of PSI reduced to 1-carboxy-3-deoxy-octitol. The reduced PSI was checked by one-dimensional NMR. The data confirmed the PS structure but also revealed de-O-acetylation, which occurred during the reduction procedure (data not shown). The PS in the conjugate PSI–BSA represented the O-specific PS devoid of a major part of the core OS region. The total carbohydrate:protein ratio in the PSI–BSA was 1:1 and was determined with the use the phenol-sulfuric acid procedure (Fox and Robyt 1991) combined with the protein concentration measurement (A_{280 nm}). Molecular weight of the conjugate was estimated by SDS–PAGE analysis with the use of protein molecular weight markers within the range 40–200 kDa (Supplementary data, Figure S3). Two methods were used for the detection of the PSI–BSA: Coomassie staining for the detection of BSA (weak staining due to the PS substitution) and immunoblotting with the use of polyclonal antibodies (Abs) specific for the O-specific PS of LPS 1200 for the detection of carbohydrate moiety. Obtained PSI–BSA conjugate was heterogeneous mixture due to the heterogeneity of used PSs (the number of RUs). Estimated mass of the glycoconjugate was within a range of 72 to >200 kDa with stronger diffused bands around 130, 170 and >200 kDa (Supplementary data, Figure S3). Due to the described heterogeneity of the PSs obtained results allowed only to estimate the number of O-specific PS RUs (molecular weight ~1071 Da) per conjugate molecule (molecular weight of BSA ~67 kDa). In the case of PSI–BSA, the calculated number was around 5 RUs (the conjugate of 72 kDa), 59 RUs (the conjugate of 130 kDa), 96 RUs (the conjugate of 170 kDa) and >120 RUs (the conjugate of >200 kDa).

**Binding of recombinant ficolin-3 to the PSI–BSA**

The conjugate of the reduced PSI and BSA (PSI–BSA) was used to assess binding between PSI1 and human serum-derived or rh ficolin-3 in ELISA. The PSI used for the preparation of the conjugate was also devoid of O-acetylated groups as was determined by NMR analysis (data not shown). The binding was compared with that of native and de-O-acetylated LPS 1200. Similar dose-dependent binding of both serum and rh ficolin-3 was demonstrated for all ligands with the strongest binding to de-O-acetylated LPS (Figure 6A and B). This showed that the presence of the ligands did not affect significantly the interaction between ficolin-3 and the O-specific PS of LPS 1200.

**Ficolin-3 selectivity for interaction with PSI–BSA and LPS 1200**

In order to compare the specificity of binding of ficolin-3 to *H. alvei* 1200 LPS, PSI–BSA and BSA-NAc, an ELISA analysis was performed (Figure 7). A very strong reactivity of ficolin-3 with *H. alvei* 1200 LPS and PSI–BSA was confirmed. These products were not recognized by other ficolins (1 and 2) or MBL. However, the human sera used in this experiment contained some natural Abs reacting with native LPS and PSI–BSA. None of the lectins recognized BSA, and only ficolin-3 reacted weakly with BSA-NAc (Figure 7). Slightly higher A_{405 nm} values for the ficolin-3/BSA-NAc interaction were recorded when the N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES) buffer recommended by Munthe-Fog et al. (2009) was used (data not shown). These results showed that *H. alvei* 1200 LPS or PSI–

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**Table I.** Interpretation of MALDI-TOF mass spectra of fractions PS4, PS5 and OS1 isolated from LPS *H. alvei* 1200

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<th>Observed ion (m/z)</th>
<th>Interpretation of ion</th>
<th>Schematic structure of molecular ion (M)</th>
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<th>Fraction OS1</th>
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<td>1380.99</td>
<td>[M–H]–</td>
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<td>3200.54</td>
<td>[M–H]–</td>
<td>RU–RU–RU–[Hep-Kdo] + 1 × OAc</td>
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*a/m/z values correspond to monoisotopic mass; RU, a complete repeating unit of the O-specific PS; RUc, an incomplete RU devoid of terminal α-D-GlcP; OAc, O-acetyl group.

*bIons corresponded to core OSs (core OS = 2 × Hex + 3 × Hep + Kdo + 3P + EtN; Jachymek et al. 1995).
BSA dissolved in MBL-binding buffer can be used as specific and selective ligands for ficolin-3 detection. We have also performed an inhibition test for ficolin-3-LPS 1200 reactivity with monosaccharides, such as D-Gal, D-Man, D-Glc, D-GlcNAc and L-Fuc. Unlike LPS 1200 or isolated PS1 used as inhibitors, there were no significant inhibitory effects of tested monosaccharides, even in high doses (up to 250 mM; data not shown). Lipooligosaccharides (LOS) isolated from deep rough mutants of Salmonella (Rd1-Rd2) were used to examine the role of terminal Hep in interaction between the O-specific PS 1200 and ficolin-3. Core OSs of Salmonella Rd1 and Rd2 LOSs are built of Kdo and Hep residues only (Galanos et al. 1977; Clas and Loos 1981). The lack of interaction observed in immunoblotting and ELISA of ficolin-3 with these LOSs (data not shown) excluded the role of terminal heptose as a ligand for ficolin-3.

*Estimation of ficolin-3 concentration and ficolin-3/MASP-2 complex activity in human sera*

We have demonstrated that recombinant ficolin-3 binds both LPS 1200 and PS1–BSA in a dose-dependent manner (Figure 8). Therefore, either can be used to estimate ficolin-3 concentrations in sera. First, ficolin-3 content was determined in sera A and B, giving values of 27.5 and 7.5 µg/mL, respectively. The values obtained were slightly lower than these measured with a “sandwich” ELISA (see Materials and methods). The differences could be explained by the detection of total protein concentration in the “sandwich” ELISA and active molecules only in the method proposed here. Using PS1–BSA as the solid phase, 73 sera of healthy adult volunteers were tested. The mean ficolin-3 level equaled 17.4 µg/mL (median: 16.4 µg/mL, range: 7.4–34.7 µg/mL; Figure 8A). Serum A was used as a standard. Furthermore, this enabled us to employ serum A as a standard for the estimation of ficolin-3-dependent lectin pathway activity, measured by C4b deposition. The value for that serum was arbitrarily determined as 1 U/mL. Mean activity for the healthy volunteers was 897 mU/mL (median: 868 mU/mL; range: 421–1616 mU/mL; Figure 8B). A statistically significant correlation between ficolin-3 concentrations and activities was observed ($r = 0.55; P < 0.0001$; Figure 8C).

*Discussion*

Numerous plasma proteins bind the lipid A–inner core region of LPS, contributing to its clearance from circulation or
delivery to cellular receptors thus leading to the modulation of its biological activity. The O-specific PS, being its most variable region, is a target for specific Abs and complement components, including MBL (Joiner et al. 1986; Jiang et al. 1995). For example, the interaction of MBL with LPS could lead to serious pathological consequences such as anaphylactoid shock or rapid death, as was shown in a murine model (Zhao et al. 2002; Swierzko et al. 2003).

Herein, we have identified a well-defined group of new ficolin-3 ligands among LPSs of *H. alvei*, capable of activating the LP of complement. Both native and recombinant ficolin-3 specifically recognized the O-specific PSs of identified LPSs. This interaction was demonstrated with both isolated LPS and LPS present on the surface of bacteria, suggesting an important role for ficolin-3 as a first-line of defense molecule during infection. The observed binding was limited to the O-specific PSs and the Hep-Kdo disaccharide present at the reducing end of the O-specific PSs did not take a part in this interaction.

The complexity of ligands such as LPS or EPS and the lack of precise structural information make the identification of structural elements recognized by ficolin-3 difficult. Data from inhibition tests using monosaccharides did not give clear results. Certain effects were reported previously in the case of D-GlcNAc, D-GalNAc, D-Gal and D-Fuc (Endo et al. 2007; Garlatti et al. 2007, 2009; Matsushita 2007, 2009). However, the interaction of ficolin-3 with EPS or whole cells of *A. viridans* was not prevented by any of these sugars (Tsujimura et al. 2001; Krarup et al. 2004). Recently, Gout et al. (2010) using glycan array screening found no potent ligand for ficolin-3 among 377 compounds, including numerous mono-, oligo- or PSs. On the other hand, they observed an interaction of ficolin-3 with BSA-Gal conjugate (in contrast to BSA-GlcNAc and BSA-GalNAc). We have not found significant inhibitory potency of such monosaccharides as D-Gal, D-Man, D-Glc, D-GlcNAc and L-Fuc.

X-ray data presented by Garlatti et al. (2007) and obtained for crystals of the fibrinogen-like recognition domain of ficolin-3 in complex with ligands such as d-Gal, d-Fuc and l-Fuc allowed for the identification of major binding sites and excluded l-Fuc as a ligand. According to their studies, this site forms an external part of the trimer, in the vicinity of the Ca$^{2+}$-binding site and is homologous to the GlcNAc-binding site identified in horseshoe crab tachylectin 5A (TL5A). However, contrary to TL5A, its spatial structure and non-conservative character of a few key amino acids allow for
some flexibility of binding site (Garlatti et al. 2007). The crucial role of Ca$^{2+}$ suspected previously by Garlatti et al. (2007) was further evidenced by Hein et al. (2010) for a binding of ficolin-3 to BSA-NAc and subsequent complement activation. However, an addition of magnesium ions did not allow ficolin-3 to recognize BSA-NAc (Hein et al. 2010). According to our observation, the interaction between ficolin-3 and LPS 1200 requires the presence of both calcium and magnesium ions, as was shown by a strong inhibition of binding of ficolin-3 to LPS by EDTA or EGTA and much weaker in the case of EGTA/Mg$^{2+}$ (Figure 2C).

Crystallographic analysis done by Garlatti et al. (2007) was also performed for ficolin-2 and monosaccharide ligands. Further studies of ficolin-2 binding to more complex ligand (1,3-β-D-glucan, built of four Glc residues) revealed a novel extended recognition area with three additional binding sites involved. The shape of the recognition area allowed for binding of PSs and provided the observed plasticity of recognition by ficolin-2. Due to the lack of a proper, structurally characterized ligand for ficolin-3, similar analysis has not been performed for ficolin-3 and its natural ligands such as EPS of A. viridans. Despite of some differences in structure, amino acids composition and ligand specificity between ficolin-2 and ficolin-3, ficolin-3 can also have similar affinity toward complex ligands characterized by repetition pattern, especially at the surface of microorganism.

According to the previous studies, it could be assumed that the presence of d-GlcNAc, d-GalNAc, d-Gal and d-Fuc may determine the binding specificity of ficolin-3 (Endo et al. 2007; Garlatti et al. 2007, 2009; Matsushita 2007, 2009). Moreover, the example of BSA-NAc supports a conclusion about an important role of the N-acetyl groups. However, the affinity for acetylated compounds, including BSA-NAc and HSA-NAc (acyetylated-human serum albumin), was also reported for ficolin-2 and ficolin-1 (Frederiksen et al. 2005; Ng et al. 2007; Lacroix et al. 2009; Munthe-Fog et al. 2009).

The advantage of PS1 is a much stronger LP activation in comparison to BSA-NAc. Moreover, the O-specific PSs identified represent components of the natural target for this lectin and are not recognized by other ficolins or MBL. Some structures of H. alvei O-specific PSs, identified here as exclusive ligands for ficolin-3, are known (Supplementary data, Figure S1; Katzenellenbogen et al. 1992, 1999; Dag et al. 2004). Their comparison clearly shows that the presence of d-GlcNAc, d-Quip4NAc and O-acetyl groups is a common feature of the O-specific PSs isolated from LPS 1200, 1203, 1205 and 23. However, no substantial difference in the reactivity of ficolin-3 with native and de-O-acetylated LPS 1200 was observed. Therefore, the O-acetyl groups do not constitute a factor for this interaction. It might be speculated that the disaccharide, $\alpha$-D-GlcNAc-(1→3)-β-D-Quip4NAc-(1→, could be a common fragment in 23, 1200, 1203 and 1205 LPSs, which is recognized by ficolin-3. Moreover, the presence of N-acetyl groups is the only structural feature shared by all presented here O-specific PSs and BSA-NAc. Therefore, it suggests that the structural arrangement of N-acetyl groups may determine the pattern recognition. Recently, Thomsen et al. (2011) have hypothesized
differences in binding patterns of all human ficolins result from different spacing of acetyl groups in the used ligands or are due to oligomerization defects observed for recombinant proteins. It should be stressed that there are some other “N-acetylated” LPSs among *H. alvei* LPSs which were not bound by ficolin-3 (e.g. LPS 1192) as was shown by dot blotting, ELISA and flow cytometry.

An advantage of the described PSs is their selectivity toward ficolin-3. We have shown previously that LPSs of *H. alvei* belong to the group of atypical endotoxins, due to the presence of the Kdo residue in the outer core region (Lukasiewicz et al. 2009). This feature, characteristic for most of *H. alvei* LPSs, including LPS 1200, 1203 and 1205 (Lukasiewicz et al., unpublished results), makes possible easy isolation of the O-specific PS devoid of large part of the core region, which could be bound by other immune factors such as MBL (Figure 1C) or immunoglobulins. This feature prompted us to prepare PS1–BSA neoglycoconjugate as a tool to measure ficolin-3 concentration in human serum as well as ficolin-3/MASP-2-dependent complement LP activity (in the C4b deposition assay), without interference from MBL, ficolin-2 and ficolin-1 or natural Abs. It may also be used for the isolation of ficolin-3 from body fluids. The employment of hypertonic MBL-binding buffer (Petersen et al. 2001) for both concentration and complement activation assays seems to be more reasonable than HEPES buffer (Munthe-Fog et al. 2009), due to higher values of absorbance. Moreover, the MBL-binding buffer prevents classical complement pathway activation (Petersen et al. 2001), which is extremely important when C4b deposition is determined. According to previous studies, the average concentration of ficolin-3 in human sera is within the range 20–30 µg/mL (Krarup et al. 2005; Munthe-Fog et al. 2008, 2009; Andersen et al. 2009). The methods previously used employed monoclonal Abs (mAbs) (“sandwich ELISA”; Krarup et al. 2005; Munthe-Fog et al. 2008, 2009; Andersen et al. 2009) or *A. viridans* (Kristof et al. 2003) EPS as a solid phase for ELISA. Until now there is no “golden standard” for the determination of both ficolin-3 level and activity. We present an alternative, functional assay useful for both scientific and diagnostic purposes.

The exploration of the relationship between innate immunity mechanisms and disease/risk factors depends on appropriate experimental tools allowing the isolation, purification as well as the measurement of concentration and specific activity. This is difficult in the case of such a complex system as complement, involving several key molecules (immunoglobulin G (IgG)/immunoglobulin M (IgM), MBL, ficolin-1, ficolin-2 and ficolin-3 etc.) with wide specificity. Genes encoding MBL and ficolins are characterized by polymorphisms, which are correlated with function and also changes in a specific lectin concentration. Thus, other approaches based on a specific, functional ligand for ficolin-3 could give results correlated with functional and effective form of these lectins.

**Materials and methods**

**Bacterial strains**

*Hafnia alvei* strains PCM 1, 1M, 2, 17, 23, 31, 32, 37, 38, 39, 399, 481, 537, 600, 744, 974, 2694, 1187, 1188, 1190, 1191, 1192, 1195, 1196, 1198, 1200, 1203, 1204, 1205, 1206, 1207, 1208, 1209, 1210, 1211, 1212, 1213, 1214, 1215, 1218, 1220, 1221, 1222, 1224 and 114-60 were obtained from the PCM at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The bacteria were grown in Davis medium, killed with 0.5% phenol and centrifuged using a CEPA flow laboratory centrifuge (Petersson et al. 1997).

**Abs and sera**

mAbs, mouse anti-human MBL (clone HYB 131-01) and mouse anti-human ficolin-1 (clone ABS-036-01), came from BioPorto, Denmark. Mouse anti-human ficolin-3 mAb (clone 4H5) and anti-human ficolin-2 mAb (clone 2F5) were prepared by Misao Matsushita. Polyclonal rabbit anti-human C4 Abs were obtained from Sigma, US. Secondary Abs, horseradish peroxidase (HRP)- or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig, HRP-conjugated rabbit anti-human IgG, A and M and HRP-conjugated goat anti-rabbit Ig, were obtained from DAKO, Denmark.

Normal human sera (A and B) were collected from healthy adult volunteers. Serum A had normal ficolin-3 and ficolin-2 levels (32.7 and 3.5 µg/mL, respectively) and low MBL concentration (0.2 µg/mL). Serum B had low ficolin-3 and ficolin-2 levels (12 and 2 µg/mL, respectively) and normal MBL concentration (2 µg/mL). The levels of ficolin-3 and ficolin-2 in these sera were kindly measured (by “sandwich” ELISA) by Prof. Jens C. Jensenius (Department of Medical Microbiology and Immunology, University of Aarhus, Denmark) and Dr. David C. Kilpatrick (Scottish National Blood Transfusion Service, National Science Laboratory, Edinburgh, Scotland), respectively. MBL concentrations were determined as described previously (Cedzynski et al. 2004). Ficolin-1 was not determined in either serum. Normal human sera tested for ficolin-3 concentration and ficolin-3/MASP complex-dependent complement activity were obtained from 73 healthy adults. The local ethical committee approved the study and samples were obtained with written informed consent of the volunteers.

**Preparation of the BSA-NAc**

The BSA-NAc was prepared as described by Munthe-Fog et al. (2009).

**Preparation of LPSs and O-specific PS**

LPSs were extracted from bacterial cells by the hot phenol/water method (Westphal and Jann 1965) and purified as described previously (Pettersson et al. 1997). Poly- and OSs were isolated by mild acidic hydrolysis and fractionated as described elsewhere (Dag et al. 2004).

**De-O-acetylation of LPS**

LPS 1200 was suspended in 12.5% NH₃ in water and incubated for 16 h at 22°C, followed by dilution with water and lyophilization.
The PS1 (22 mg/mL of H₂O) was reduced with NaBH₄ and the mixing time for NOESY and ROESY was 200 and 60 and 100 ms. The delay time in HMBC was 60 or 65 ms, clean-TOCSY experiments, the mixing times used were 30, 60 and 100 ms. The data were acquired and processed using standard Bruker software and assigned with the use of separate experiment. The data were acquired and processed referenced to an external 85% phosphoric acid (NMR spectroscopy experiments, phosphorus resonances were referenced to an external 85% phosphoric acid (δ 0 ppm) in a separate experiment. The data were acquired and processed using standard Bruker software and assigned with the use of SPARKY software (Goddard and Kneller 2001). The signals were assigned by two-dimensional experiments (correlation spectroscopy (COSY), clean-total correlation spectroscopy (TOCSY), NOESY, ROESY, HMBC, HSQC-DEPT and HSQC with and without carbon decoupling). In the clean-TOCSY experiments, the mixing times used were 30, 60 and 100 ms. The delay time in HMBC was 60 or 65 ms, and the mixing time for NOESY and ROESY was 200 and 500 ms, respectively.

Reduction of the fraction PS1
The PS1 (22 mg/mL of H₂O) was reduced with NaBH₄ (10 mg) for 16 h at 37°C. The solution was acidified with CH₃COOH to pH 4.5 and purified by gel permeation chromatography, performed on a Bio-Gel P-2 column equilibrated with 0.05 M pyridine/acetate buffer at pH 5.6. The fraction containing reduced PS1 was freeze-dried, checked by one-dimensional NMR analysis and used for conjugation with BSA.

Conjugation of the reduced PS1 with BSA
The conjugation was performed according to Lonngren and Goldstein (1978). Briefly, the reduced PS1 was dissolved in H₂O, and the pH of the solution was adjusted to 4.75, followed by the addition of 1-ethyl-3-(dimethylaminopropyl)-carbodimide hydrochloride. The reaction was carried out for 1 h at 22°C, and pH 4.75 was maintained throughout by the addition of HCl. After 1 h of additional incubation, 0.4 mL of BSA solution (5 mg/mL) was added to the solution and the conjugation was carried out for 4 h at 22°C. The conjugate (PS1–BSA) was purified by gel permeation chromatography, using a Sephadex G-100 column equilibrated with 5% ethanol. The fraction of the conjugate obtained was checked for the presence of protein (A₂₈₀nm measurement) and PS1 (a dot blotting with the use of polyclonal rabbit serum against H. alvei 1200). The total carbohydrate:protein ratio in the PS1–BSA was determined with the use of protein concentration measurement (A₂₈₀nm) combined with the phenol-sulfuric acid procedure (Fox and Robyt 1991). Reduced fraction PS1 was used as a standard for phenol-sulfuric acid total carbohydrate determination. Molecular weight of the conjugate was estimated by SDS–PAGE according to the method of Laemmli (1970). The conjugate fractions were visualized by Coomassie staining and immunoblotting performed on the SDS–PAGE-separated (7.5% gel) conjugate fractions with the use of rabbit polyclonal serum against H. alvei 1200, as described previously (Lukasiewicz et al. 2009). Following protein molecular weight markers were used: M1 (prestained, 161-0309, Bio-Rad, US), M2 (prestained, SM0671, Fermentas, Lithuania) and M3 (unstained, SM0661, Fermentas, Lithuania).

Western blotting and dot-blot analysis
Hafnia alvei LPSs were separated on 15% polyacrylamide gels (Laemmli 1970) and then transferred onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with Superblock solution (ThermoScientific, US) and incubated overnight (at 4°C) with serum A (for ficolin-3 and ficolin-2 detection) or serum B (for MBL detection), 30-fold diluted in a high-ion strength MBL-binding buffer (20 mM Tris/HCl, 10 mM CaCl₂, 1 M NaCl, pH 7.4; Petersen et al. 2001), supplemented with 0.1% BSA or a pool of sera A and B (1:1, each prediluted 1:15 in the same buffer, for ficolin-1 detection). Then, primary Ab (anti-MBL or anti-ficolin-3 or anti-ficolin-2 or anti-ficolin-1, respectively) was added, followed by the HRP-conjugated rabbit anti-mouse Ig, as a secondary Ab. Membranes were incubated with electrochemical luminescence (ECL) substrate (Santa Cruz Biotechnology, US) and then exposed to an X-ray film. For dot-blot analysis, 10 µg of the tested LPS was applied directly on the nitrocellulose membrane (Schleicher and Schuell, Germany). After blocking, the membrane was incubated with serum A as a source of ficolin-3 and the binding of ficolin-3 to LPS was detected as for Western blotting.

Binding of ficolin-3 to bacterial cells
Bacterial cultures were washed out from the solid medium with PBS, centrifuged and fixed with 1.5% formaldehyde. After washing with cold PBS, cell pellets were suspended in veronal-buffered saline supplemented with 0.1% gelatin, 0.3 mM CaCl₂ and 2 mM MgCl₂ (GVB²⁺, Gelatin Veronal Buffer) to OD = 1 (A₆₀₀nm). The suspension (100 µL) was centrifuged. The pellet was re-suspended in 50 µL of GVB²⁺ buffer and then incubated with or without 1 µg of human recombinant ficolin-3 (R&D Systems, US) for 1 h at 37°C. The bound protein was detected with mouse anti-human ficolin-3 mAb followed by FITC-labeled anti-mouse Ig Ab. The reactivity was estimated using Cytomics FC 500 MPL Beckman-Coulter flow cytometer.

Inhibition of binding of ficolin-3 to bacterial cells
ELISA inhibition was performed by analogy to the method described in section Inhibition of ficolin-3 interaction with LPS by calcium and magnesium chelators with the following modifications. Human serum (serum A, prediluted 1:50) was incubated for 30 min at 28°C with different amounts of H. alvei 1200 LPS. Next, the mixture was transferred to the wells of microtiter plate coated with suspension of H. alvei 1200
cells in PBS (10 µg/well). After 2 h incubation at 37°C, the bound H-ficolin was detected with the use of H-ficolin-specific mAb and HRP-labeled anti-mouse Ig. Absorbance (A_{405 nm}) was measured with the use of a Benchmark Plus microplate spectrophotometer (Bio-Rad).

**Binding of lectins and immunoglobulins to various ligands**

Microtiter MaxiSorp U96 plates (NUNC, Denmark) were coated with LPS (5 µg/well), PS1–BSA (1 µg/well), BSA (0.5 µg/well) or BSA-Nac (0.5 µg/well) dissolved in PBS. After overnight incubation at 4°C, plates were washed with tris buffered saline (TBS)-Ca²⁺ containing 0.05% Tween-20 and blocked with 0.1% BSA in TBS-Ca²⁺ (2 h, 37°C). Human serum (A or B) or human recombinant ficolin-3 was diluted in MBL-binding buffer containing 0.1% BSA, without Tween-20 and blocked with 0.1% BSA in TBS-Ca²⁺ (2 h, 37°C). Human serum (A or B) or human recombinant ficolin-3 was diluted in MBL-binding buffer containing 0.1% BSA, without Triton X-100. Plates were incubated overnight at 4°C. Then, to estimate ficolin-3 binding, specific mAbs (0.5 µg/mL in 0.1% BSA/TBS-Ca²⁺) were added and after 2 h at 37°C followed by detection with HRP-conjugated rabbit anti-mouse Ig. ABTS (Sigma) was employed as a substrate for peroxidase. Absorbance (A_{405 nm}) was measured with the use of a Benchmark Plus microplate spectrophotometer (Bio-Rad). Alternatively, to test the binding of ficolin-2, ficolin-1, MBL and immunoglobulins appropriate primary and corresponding HRP-conjugated secondary Abs were employed.

**Inhibition of ficolin-3 interaction with LPS by calcium and magnesium chelators**

Inhibition of ficolin-3 interaction with LPS by Ca²⁺ and Mg²⁺ chelators was tested according to the modified procedure described by Hein et al. (2010). Briefly, microtiter MaxiSorp U96 plates (NUNC, Denmark) were coated with H. alvei 1200 LPS (5 µg/well), dissolved in PBS. After overnight incubation at 4°C, plates were washed with TBS-Ca²⁺ containing 0.05% Tween-20 and blocked with 0.1% BSA in TBS-Ca²⁺ (2 h, 37°C). Human serum (A or B), prediluted 1:25 was added and after 2 h at 37°C followed by detection with HRP-conjugated rabbit anti-mouse Ig. ABTS (Sigma) was employed as a substrate for peroxidase. Absorbance (A_{405 nm}) was measured with the use of a Benchmark Plus microplate spectrophotometer (Bio-Rad).

**Ficolin-3 concentration in human sera**

In general, the procedure followed that described in the section **Binding of lectins and immunoglobulins to various ligands**. Briefly, the plates were coated with PS1–BSA diluted in PBS. After overnight incubation at 4°C, plates were washed with TBS-Ca²⁺/0.05% Tween-20 and blocked with 0.1% BSA/TBS-Ca²⁺. Sera to be tested (100-fold diluted in 0.1% BSA/MBL-binding buffer) were added and incubated overnight at 4°C. Next, low ficolin-3 serum (B), prediluted 1:3000 in 0.1% BSA/TBS-Ca²⁺, was added as a source of C4 factor. After incubation for 2 h at 37°C, any deposited C4b was detected with the use of anti-human C4 Ab and HRP-labeled goat anti-rabbit Ig. The MBL-binding buffer was used in this experiment to prevent the activation of complement via the classical pathway (Petersen et al. 2001).

**Statistical analysis**

Correlation was tested with Spearman’s test. P-values of <0.05 were considered as statistically significant.

### Supplementary data

Supplementary data for this article is available online at [http://glycob.oxfordjournals.org/](http://glycob.oxfordjournals.org/).

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### Conflict of interest

A.S., J.L., M.C, A.M., W.J., T.N., C.L. and Ludwik Hirszfeld Institute of Immunology and Experimental Therapy and Institute for Medical Biology have a patent pending on the use of identified ficolin-3 ligands as a tool for both scientific and diagnostic purposes. The authors have no additional financial interests.
Abbreviations

BSA, bovine serum albumin; BSA-NAc, N-acetylated BSA; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; ECL, electrochemical luminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis-(2-aminoethyl)ether)-N,N,N',N'-tetraacetic acid; FITC, fluorescein isothiocyanate; GVB, Gelatin Veronal Buffer; HEPES, N-(2-Hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid); HMBC, heteronuclear multiple-bond correlation; HRP, horseradish peroxidase; HSA, human serum albumin; HSQC, heteronuclear single quantum coherence; IgG, immunoglobulin G; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MASP, MBL-associated serine protease; MBL, mannann-binding lectin; MS, mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; OD, optical density; OS, oligosaccharide; PAMP, pathogen-associated molecular pattern; PCM, Polish Collection of Microorganisms; PRM, pattern-recognition molecule; PS, polysaccharide; rh, recombinant human; ROESY, rotating-frame Overhauser effect spectroscopy; RU, repeating unit; TBS, tris buffered saline; TL5A, tachylectin 5A; TOCSY, total correlation spectroscopy.

References
