The role of mannose-binding lectin in vitro and in vivo

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Mannose-binding lectin (MBL)-mediated opsonization is enhanced by the alternative pathway amplification loop

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Abstract

The complement system is a humoral effector in the innate immune system. Three activation pathways exist in the complement system, known as the classical pathway, the lectin pathway and the alternative pathway. Dysfunction of lectin pathway activation is caused by MBL deficiency.

MBL deficiency in a cohort of healthy Caucasian blood bank donors was investigated with MBL genotyping and MBL plasma concentration. Recognition of the yeast-derived zymosan by MBL was investigated with western blot. The involvement of the alternative pathway amplification loop in enhancing MBL-mediated opsonization of zymosan was investigated in a novel opsonophagocytosis assay for flowcytometry. Sera deficient for MBL, factor D or properdin were tested, and purified MBL, factor D or properdin were used to recover opsonization.

The optimal ROC cut-off value for dividing the Caucasian cohort in MBL-sufficient and MBL-deficient was calculated at 0.7 μg/ml. Thirty-eight percent of the group had concentrations below 0.7 μg/ml. Zymosan eluates opsonized with MBL-sufficient sera contain high oligomers of MBL, while eluates from MBL-deficient donors contained hardly any MBL. The MBL-, factor D- and properdin-deficient sera showed reduced opsonophagocytosis by human control neutrophils, as compared to normal MBL-sufficient sera. This reduction in opsonization was restored to normal levels by addition of purified MBL, factor D and properdin. The absence of opsonization in the factor D- and properdin-deficient sera, but presence in normal serum after blocking with anti-C1q-F(ab)2 and anti-MBL-F(ab)2, demonstrates the involvement of the amplification loop in MBL-initiated zymosan opsonization, even at very low serum concentrations (up to 3% [vol/vol]).

In conclusion, our data demonstrate that the MBL-mediated route of complement activation depends on the alternative pathway amplification loop for optimal opsonization of zymosan.

Introduction

Complement deficiencies can lead to severe infectious-related problems. Complement consists of a group of more than 35 proteins, which interact to recognize, opsonize and/or kill invading micro-organisms or altered host cells (e.g. apoptotic or necrotic cells). Soluble complement proteins make up about 5% of the total protein content of human blood plasma. They are also present at lower concentrations in other body fluids.

There are three activation pathways in the complement system, known as the classical pathway, the lectin pathway and the alternative pathway. Recognition proteins such as Mannose-
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binding lectin (MBL) and ficolins bind to repeating sugar residues on target cells, initiating the lectin pathway of complement activation. MBL is a plasma protein that belongs to the collectin family. It has a bouquet-like structure, comprised of several subunits (1-3). These subunits are each composed of three identical polypeptides with a carbohydrate recognition domain (CRD) and a collagenous region. The CRDs bind in a calcium-dependent manner to various carbohydrate structures on microbial surfaces, such as viruses, protozoan parasites, fungi, and various bacteria (4-7).

Dysfunction of lectin pathway activation is caused by MBL deficiency, which is suggested to result in increased risk and severity of infections. Blood levels of MBL are affected by three single nucleotide polymorphisms (SNPs) in codon 52, 54, and 57 (D, B, and C variants, respectively) of exon 1 of the \textit{MBL2} gene (3, 8). The normal allele is called A and the common designation for the variant alleles is O. Three promoter polymorphisms (H/L, X/Y, and P/Q at codons -550, -221, and +4, respectively) are in linkage disequilibrium with the three dominant exon-1 SNPs, resulting in seven possible haplotypes: HYPA, HYPD, LYPA, LYPB, LYQA, LYQC, and LXPA (9, 10). The HYA haplotypes induce high MBL concentrations, whereas exon 1 mutations (O variants) and the LXA haplotypes cause reduced MBL plasma concentrations (11). Therefore, donors can be classified into high (HYA/HYA, HYA/LYA, HYA/LXA, LYA/LYA and LYA/LXA), medium (LXA/LXA, HYA/O and LYA/O) and low (LXA/O and O/O) MBL expression groups (9, 12). The frequency of the haplotypes differs between ethnic groups (12). During opsonization of micro-organisms, high oligomers of MBL associate with various MBL-associated serine proteases (MASP-1, -2 and -3) to generate a functional unit for complement activation (1-3, 13).

Recognition of opsonized micro-organisms by the classical pathway, via antibodies that bind to C1q, and the lectin pathway, via MBL-MASP complexes, leads to activation of serine proteases that cleave complement components C4 and C2. This leads to the formation of the protease complex C4b2a, which then cleaves C3 into C3a and C3b. C3b is a central component of the complement system due to its many effector functions (14).

In contrast to the classical and lectin pathways, the alternative pathway is activated by a mechanism targeting surface clusters of both charge and neutral sugar (15). There is continuous spontaneous complement activation known as the amplification loop: C3 undergoes hydrolysis of its internal thiol-ester at a low rate to form C3i [or C3(H_2O)]. When C3i is in complex with the serine protease factor B, factor B is cleaved by the serine protease factor D to form C3iBb. Bb in the complex cleaves C3 to form C3b that binds randomly and covalently to any nearby cell or macromolecular surface. Once deposited, this C3b can bind more factor B, which is cleaved again by factor D, forming the alternative pathway C3 convertase C3bBb stabilized by properdin, thus causing more C3b to be fixed to the target surface. This continuous activation of the amplification loop in the alternative pathway ultimately produces strong opsonization.
Roos et al. (16) found that in MBL-deficient individuals, the antibody-mediated classical pathway activation can compensate for impaired MBL-mediated target opsonization. Although the involvement for the alternative pathway in complement-dependent hemolysis via the lectin pathway has been suggested (17), the role of the alternative pathway amplification loop in MBL-mediated opsonization has remained unclear. To study this involvement, we used zymosan, which is prepared from the *Saccharomyces cerevisiae* cell wall as test particles, because it consists of protein-carbohydrate complexes that are recognized by MBL.

The involvement of the alternative pathway amplification loop in enhancing MBL-mediated opsonization of zymosan was investigated. We used an opsonophagocytosis assay with sera deficient for the alternative pathway proteins factor D or properdin to test the involvement of these proteins in MBL-mediated opsonization and subsequent phagocytosis of zymosan by normal human neutrophils. Our data show that the amplification loop of the alternative pathway is of critical importance in the MBL-mediated complement-dependent opsonization of zymosan.

**Materials and methods**

*Serum samples and complement assays*

Aliquots of human serum from 23 MBL-sufficient donors, with MBL concentrations ≥0.7 μg/ml, 21 MBL-deficient donors, with MBL concentrations <0.7 μg/ml, 2 related factor D-deficient donors (18), a C2-deficient donor and a properdin-deficient donor were obtained with informed consent. All aliquots were stored at -80°C until tested.

The activities of the classical (CH50) and alternative (AP50) pathways of the complement system were detected as described (19). Factor D-deficiency was determined by ELISA technique, with purified factor D (Quidel Corp., San Diego, CA, USA) as a standard (18). Deficiency of C2 was detected by restoration of hemolytic activity of the deficient serum after addition of purified C2 (Serva, Heidelberg, Germany), as described (15). Deficiency of properdin was detected by the Ouchterlony technique. Purified properdin was kindly provided by M. R. Daha (Department of Nephrology, Leiden University Medical Centre, Leiden, The Netherlands). The activities of the classical, lectin and alternative pathways of complement were analyzed with the Wielisa kit (Wieslab, Sweden) (20). This ELISA system is based on three different coatings (C1q, mannann and LPS, respectively) and determination of C5-C9 as the read-out (21).

*Polymorphonuclear leukocyte isolation*

Fresh isolates of polymorphonuclear leukocytes (PMN) were purified from whole blood by centrifugation over a Percoll gradient as described by Roos (22). After lysis of the erythrocytes,
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the PMN were washed in phosphate-buffered NaCl and adjusted to 10^7/ml in HEPES medium (132 mM NaCl, 6 mM KCl, 1 mM MgSO_4, 1.2 mM KH_2PO_4, 20 mM HEPES, 2 mM CaCl_2, 5.5 mM glucose and 0.5% [wt/vol] human serum albumin, pH 7.4).

**MBL purification and MBL assays**

MBL was prepared from human serum as described previously by Kilpatrick (23). The MBL concentration was determined by ELISA and the purity was verified by denaturing SDS-PAGE. The purified MBL was contaminated by IgM (11%) and IgA (1%) (24).

Functional MBL concentrations were determined in a solid-phase ELISA with mannan coated to the solid phase and detection with monoclonal antibody αMBL-1 (Sanquin, biotinylated mouse-anti-human MBL IgG, 10 µg/ml). Briefly, microtiter plates were coated with 100 µg/ml mannan in 0.1 M NaHCO₃, pH 9.6 overnight at room temperature. The microtiter plates were washed 5 times with H₂O. Plasma or serum samples and MBL standards (standard serum, 1.5 µg/ml MBL) were diluted in TTG/Ca²⁺ (20 mM Tris pH 7.4 / 150 mM NaCl / 0.02% Tween-20 / 0.2% gelatin / 10 mM CaCl₂), with 10 U/ml heparin, added to plates and incubated shaking at room temperature for 1 hour. After washing, the plates were incubated for 1 hour with biotinylated αMBL-1 in TTG/Ca²⁺, washed with H₂O and incubated shaking at room temperature for 30 minutes with streptavidin poly-HRP 1:10000 in TBS/Ca²⁺/2% milk (20 mM Tris pH 7.4 / 150 mM NaCl / 10 mM CaCl₂ / 2% milk). After washing, the color was developed with tetramethyl-3,3′,5,5′-benzidine (TMB)/0.01% H₂O₂ in 0.1 M Na-acetate pH 5.5, stopped with 2 M H₂SO₄, and measured spectrophotometrically at 405 nm (BioAssay Reader, Sunrise, Tecan, Salzburg, Austria).

Six single nucleotide Polymorphism variants (SNPs) of the MBL2 gene (AD, AB, AC, HL, XY, and PQ) were analysed with Taqman Allelic Discrimination assays (ABI Prism® 7000 Sequence Detection System, Applied Biosystems, USA). Primer and probe sequences were taken from the NCBI website (http://snp500cancer.nci.nih.gov/snplist.cfm). Probe concentration, 200 nM; primer concentration, 900 nM. The assay was performed in 15 µl, with 20 ng DNA. A pre-read was performed at 60°C. Thermocycling conditions for all assays were 2 minutes at 50°C, 10 minutes at 95°C, 50 cycli of 30 seconds at 92°C and 1 minute at 60°C (except for AB, which was performed at 62°C), followed by 10 minutes at 60°C. After the polymerase chain reaction, a post-read was performed. Fluorescent signal was corrected for pre-read fluorescent background. Analysis of the SNPs was performed with sequence detection software (ABI Prism 7000 software version 1.1, Applied Biosystems, USA).

**Preparation of antibody F(ab)₂ fragments**

One mg of blocking monoclonal antibody against C1q (αC1q-85, Sanquin) and against MBL (αMBL-1, Sanquin) (16, 25) were used to generate F(ab)₂ fragments. Antibody was dialysed against 0.1 M tri-Natrium Citrate-dihydrate, pH 3.5. Pepsin was added to a concentration of 25 µg/
ml and samples were incubated overnight at 37°C. The F(ab)_2 fragments were dialysed against PBS. F(ab)_2 preparation was checked by SDS-PAGE.

**Zymosan-FITC preparation**

Ten mg of zymosan (ICN Biochemicals) was suspended in 1 ml of PBS (pH 8.5) and was incubated for 30 minutes at 37 °C with 5 μl of FITC solution (2 mg/ml DMSO) in the dark. After washing 3 times with PBS (pH 7.4), it was resuspended in 1 ml of PBS (pH 7.4). Aliquots of zymosan-FITC were kept in the dark at –30°C.

**Opsonization**

Zymosan-FITC (250 μg) was opsonized in 250 μl of 3% (vol/vol) human serum in HEPES medium with 10 IE/ml heparin (Leo pharmaceuticals), during 30 min of incubation at 37°C while shaking. Thereafter, the opsonized zymosan was washed twice and resuspended in 25 μl of HEPES medium.

Recovery of the opsonization in deficient sera was tested by adding purified MBL, C2, D, and/or properdin (to a final concentration corresponding with plasma concentrations of 5, 3, 1 and 20 μg/ml respectively). With the use F(ab)_2 fragments of blocking monoclonal antibodies against C1q (αC1q-85, 10 μg/ml) or against MBL (αMBL-1, 5 μg/ml) the classical and lectin pathways of complement were blocked, to investigate the contribution of each complement pathway separately and in combination.

**Phagocytosis assay**

Phagocytosis was started by mixing opsonized zymosan-FITC (250 μg) with neutrophils (0.5x10^6) in HEPES medium, in a total volume of 250 μl. Incubation was performed shaking, at 37°C. At different times (0, 2, 5, 10 and 20 minutes) 50-μl samples were taken and added directly to 150 μl of ice-cold stopbuffer (20 mM NaF, 0.5% paraformaldehyde, 1% bovine serum albumin in PBS), to stop phagocytosis.

Phagocytosis was determined by flow cytometry (FACSCalibur, Becton Dickinson). Green Fluorescence (FL1) of zymosan-FITC was plotted against the cellular forward scatter (FSC). Phagocytosis was measured as the mean fluorescence intensity (MFI) multiplied by the percentage of the neutrophils gated in R2 (figure 3). Non-phagocytized zymosan was excluded from analysis by gate R1.

**Non-reduced SDS-PAGE and Western blotting**

SDS-PAGE was performed under non-reducing conditions on 5 % acrylamide gels. Samples were boiled with an equal amount of sample buffer (125 mM Tris, 20 v/v % glycerol, 5 w/v % SDS and 0.02 w/v % Coomassie blue) at 95 °C for at least 5 minutes. Samples and Precision Plus Protein™ standard (BioRad) as molecular marker were run at 50 mA in running...
buffer (25 mM Tris/0.19 M glycine/3.5 mM, pH 8.6) on the Mini Protean 3 system (BioRad, USA). Proteins from a SDS-PAGE gel were transferred to a polyvinylidene difluoride membrane (BioRad) electrophoretically in a 25 mM Tris/0.19 M glycine/20 v/v % methanol buffer. The membranes were blocked in milk/Tris buffered saline Tween-20 (TBST) (5% (w/v) milk, 10 mM Tris, 150 mM NaCl, 0.02 % (v/v) Tween-20, pH 7.4). After washing with TBST, the membranes were incubated in milk/TBST containing the primary monoclonal αMBL-1 antibody (1:2000). After washing, the membranes were transferred to TBST/milk containing the secondary antibody (1:2000). The membranes were washed with TBST and subsequently with phosphate buffered saline (PBS). The bound antibodies were detected with an ECL Western blotting reagent kit (Amersham Bioscience).

Statistics

Results from the opsonophagocytosis assay are expressed as percentage of phagocytosis compared to a positive control (zymosan-FITC opsonized with normal serum), which was set at 100%. All samples were tested in duplicate, on three different days (n=3). Data are presented as mean ± SEM. Phagocytosis of samples opsonized with sera from the same donors with addition of blocking antibodies or purified complement factors were, when applicable, compared with a paired t-test. MBL concentrations in figure 4A are expressed as median ± SD. An optimal cut-off plasma concentration for defining MBL deficiency was calculated by a receiver-operator characteristic (ROC) curve. SPSS 11.5 computer software was used.

Results

MBL deficiency

In a cohort of 194 healthy Caucasian blood bank donors, the MBL concentration in plasma and a complete MBL genotype was determined (Figure 1A). The MBL haplotypes were used to make 3 categories as described before (9, 26-28), i.e. high, medium and low MBL expression groups of 110 (57%), 52 (27%) and 32 (16%) donors, respectively (Figure 1B). The median MBL concentrations of donors in the high, medium and low expression groups were 1.65 µg/ml (Intraquartile range (IQR) 1.20-2.69 µg/ml), 0.52 µg/ml (IQR 0.40-0.92 µg/ml) and 0.04 µg/ml (IQR 0.02-0.13 µg/ml). The difference between the 3 groups is significant (P<0.001). An optimal ROC cut-off value for dividing the group into those with functional sufficiently high MBL concentrations from those with functional insufficient concentrations, corresponding with the medium and low groups, was calculated at 0.7 µg/ml. Seventy-three donors (38%) had MBL plasma concentrations below 0.7 µg/ml, defined as MBL deficient in further analysis. The remaining 62% of the group had concentration >0.7 µg/ml. These percentages of MBL deficiency and MBL concentrations in our control cohort correspond well to other population studies (9, 10, 27, 29-32).
MBL binding to zymosan

The recognition of zymosan by MBL was investigated with Western blotting. First, we opsonized zymosan as described in the Materials & Methods. The bound proteins were then eluted from the zymosan by boiling in sample buffer and the eluate was run on non-reduced 5% SDS-PAGE, next to a sample of the serum used for the opsonization of zymosan. Western blot was performed with monoclonal αMBL-1 antibody. Figure 2 shows that MBL-sufficient sera contain high oligomers of MBL and that these multimers bind to zymosan during opsonization. MBL-deficient sera contain almost exclusively the dimer MBL and showed hardly any binding to zymosan.

Phagocytosis assay

MBL-dependent opsonization was performed with 3% serum (unless mentioned otherwise) and incubation of 30 min at 37°C. Phagocytosis was determined by flow cytometry (Figure 3). All
experiments were performed in duplicate, on three days, with freshly isolated PMN. Data are expressed as % phagocytosis compared to that obtained with zymosan opsonized with a positive control serum set at 100%. (This serum showed normal activity in all three pathways of complement activation in the Wielisa assay (data not shown). These opsonization conditions were chosen to obtain optimal discrimination between MBL-deficient (<0.7 μg/ml) and MBL-sufficient (≥0.7 μg/ml) sera. At 3% (v/v) the MBL-sufficient sera showed 3.8 times more opsonization than the MBL-deficient sera while at 5% this was only 2.8 times more and at 10% serum the difference had almost disappeared (Figure 4B). Flow cytometry data were confirmed with confocal microscopy (data not shown).

Figure 2. Western blot of non-reduced gel stained for MBL. S1-3: 3 MBL-sufficient sera. ZE1-3: zymosan eluates after opsonization with S1-3. MBL: purified MBL. S4-6: 3 MBL deficient sera. ZE4-6: zymosan eluates after opsonization with S4-6. Arrows indicate the different oligomeric forms of MBL. Molecular weight is indicated in kDa. Right film was exposed to the blot for 5 minutes, left film for 20 minutes.

Phagocytosis kinetics (at 0, 2, 5, 10 and 20 minutes) of zymosan particles opsonized without serum (negative control) and with serum from either an MBL-sufficient control serum, an MBL-deficient serum, a C2-deficient serum, a factor D-deficient serum and a properdin-deficient serum, are shown in Figure 4C. The MBL concentration in the three complement-deficient serum samples appeared to be decreased (0.4 μg/ml, 0.1 μg/ml, and 0.5 μg/ml, respectively), but addition of purified MBL to these sera did not restore their opsonizing capacities (see Figure 5). Zymosan opsonized with a positive control serum was phagocytized within 10 minutes. After opsonization with the complement-deficient sera, phagocytosis of zymosan was diminished at all time points.
compared to phagocytosis of zymosan opsonized with the positive control serum.

Unopsonized zymosan (negative control) and zymosan opsonized with 3-10% heat-inactivated serum (56 °C, 30 minutes) were not phagocytized by human neutrophils (data not shown), suggesting that Fcg-receptor-mediated phagocytosis of zymosan opsonized in 3-10% serum did not play a major role in our assay.

**MBL-sufficient and MBL-deficient sera**

The average phagocytosis of zymosan opsonized with MBL-sufficient sera was 84 ± 5% (Figure 5A). Blocking the lectin pathway with αMBL reduced the phagocytosis of zymosan to 35 ± 6% (p<0.05), and after blocking the classical pathway with αC1q phagocytosis was diminished to 57 ± 4% (p<0.01). Blocking both pathways led to a remaining phagocytosis of 12 ± 3%.

Phagocytosis of zymosan opsonized with sera from MBL-deficient sera was 34 ± 3%, comparable with the results obtained with MBL-sufficient sera in the presence of αMBL (Figure 5B). Phagocytosis after opsonization in the presence of αC1q, or αMBL and αC1q combined, was diminished to 17 ± 2% and 13 ± 3%, respectively. Addition of purified MBL (5 µg/ml, final concentration) to the MBL-deficient sera during opsonization restored phagocytosis to 85 ± 5% (p<0.001), comparable with the opsonophagocytosis with MBL-sufficient sera.

**Factor D-deficient sera**

The involvement of the alternative pathway in MBL-mediated opsonization of zymosan was investigated with two factor D-deficient sera (18). MBL was deficient in these sera at a level of 0.1 and <0.1 µg/ml respectively. Phagocytosis of zymosan opsonized with the factor D-deficient sera was impaired (18 ± 10%; Figure 5C).

Addition of purified factor D (1 µg/ml, final
MBL-mediated opsonization enhanced by alternative pathway

![Graph A](image)

**Figure 4A-C.** Phagocytosis of zymosan by human neutrophils. (A) Zymosan opsonized with 3% [vol/vol] sera, from donors with wildtype MBL (A/A, n=23, MBL concentration 2.42 ± 1.57 µg/ml), with a heterozygous exon 1 mutation (A/O, n=13, MBL concentration 0.39 ± 0.50 µg/ml) and homozygous exon 1 mutations (O/O, n=8, MBL concentration 0.05 ± 0 µg/ml). Results are expressed as mean phagocytosis ± SEM (n=3). (B) Opsonization of zymosan with 1, 3, 5, and 10% [vol/vol] MBL-sufficient serum (●) and MBL-deficient serum (○). (C) Comparison of phagocytosis kinetics of zymosan opsonized with MBL-sufficient serum (▲), MBL-deficient serum (●), properdin-deficient serum (△), C2-deficient serum (□), factor D-deficient serum (○), or PBS, as negative control (■). (B-C) Phagocytosis was measured after t = 0, 2, 5, 10 and 20 minutes by FACS analysis. Results are expressed as a mean MFI multiplied by the percentage of the neutrophils gated in R2 ± SEM (n=3).

Addition of MBL alone had only little effect (11 ± 3%), because these sera are MBL-deficient and already showed impaired lectin pathway activation. Restoring the alternative pathway in the presence of the blocking αC1q or combination of both αC1q and αMBL resulted in a complete absence of phagocytosis (< 1%). Addition of αMBL alone had only little effect (11 ± 3%), because these sera are MBL-deficient and already showed impaired lectin pathway activation. Restoring the alternative pathway in the presence of the blocking αMBL and αC1q by addition of purified factor D to the factor D-deficient sera led to an increase in phagocytosis to 58 ± 18%.

**Properdin-deficient serum**

The role of the alternative pathway in MBL-mediated opsonization of zymosan was further investigated with a properdin-deficient serum (Figure 5D). MBL was deficient in this serum at a level of 0.5 µg/ml. Opsonization of zymosan concentration, purified MBL (5 µg/ml, final concentration) or both to the factor D-deficient sera restored opsonization to 66 ± 7% (p<0.02), 58 ± 11% and 121 ± 9% (p<0.001) phagocytosis, respectively. Opsonization with these factor D-deficient sera in the presence of the blocking αC1q or combination of both αC1q and αMBL resulted in a complete absence of phagocytosis (< 1%).
with the properdin-deficient serum showed an impaired phagocytosis of 27 ± 4%.

Addition of purified properdin (30 µg/ml, final concentration), purified MBL or both to the properdin-deficient serum during opsonization, enhanced phagocytosis to 80 ± 13% (p=0.07), 56 ± 6% (N.S.) and 102 ± 3% (p<0.05), respectively. Opsonization with this serum was decreased in the presence of blocking αC1q and αMBL (12 ±<1%) or αMBL (7 ±<1%) and completely absent in the presence of both αC1q and αMBL (<1%). Restoring the alternative pathway in the presence of blocking αMBL and αC1q antibodies by addition of purified properdin to the serum resulted in an increase in phagocytosis up to 29 ± 2%.
MBL-mediated opsonization enhanced by alternative pathway

**Alternative pathway activation**

To verify that alternative pathway activation contributes to the opsonophagocytosis in the standard use of 3% vol/vol of sera, we performed the assay with 1-10% of the sera. In Figure 6 data are shown for 3 and 10% serum. These experiments were performed in the absence (opsonization by all three pathways) or the presence of both blocking αC1q and αMBL (opsonization only by the alternative pathway). The standard condition of 3%-serum opsonization with positive control serum was set at 100% opsonophagocytosis.

At 10% serum, blocking with αMBL and αC1q decreased the phagocytosis of zymosan opsonized with an MBL-sufficient serum from 284 ± 78% to 186 ± 57% and with an MBL-deficient serum from 245 ± 38% to 166 ± 23%. In the properdin-deficient serum opsonization and phagocytosis decreased from 68 ± 28% to <1% and in the factor D-deficient sera from 175 ± 24% to <1%. The differences are caused by the amplification loop activation, which is completely absent in the properdin- and factor D-deficient sera. Again, when complement was inactivated by prior 56°C-heat treatment for 10 min, the opsonophagocytosis was completely eliminated under all condition tested (data not shown).

![Figure 6](image.png)

**Figure 6.** Phagocytosis by human neutrophils of zymosan opsonized by 3% and 10% [vol/vol] serum in absence and presence of blocking F(ab)2 fragments of αMBL and αC1q MoAbs. Results are expressed as a mean percentage ± SEM (n=3) compared to opsonization of zymosan with 3% MBL-sufficient serum (set at 100%). Zymosan was opsonized as indicated with PBS (negative control); MBL-sufficient serum; MBL-sufficient serum with blocking MoAbs; MBL-deficient serum; MBL-deficient serum with blocking MoAbs; properdin-deficient serum; properdin-deficient serum with blocking MoAbs; factor D-deficient serum; factor D-deficient serum with blocking MoAbs. Blocking MoAbs were added to a final concentration of 5 μg/ml αMBL and 10 μg/ml αC1q in 3% [vol/vol] serum and 16.6 μg/ml αMBL and 33.4 μg/ml αC1q in 10% [vol/vol] serum.

The same trend was seen in a subsequent series of experiments at 3% serum. The phagocytosis of zymosan particles opsonized with homozygous MBL-sufficient sera (set at 100%), MBL-deficient sera (22 ± 8%), a properdin-deficient serum (7 ± 6%) and a factor D-deficient sera (22 ± 11%) was found to be inhibited by addition of blocking αC1q and αMBL, to 17 ± 14%, 12 ± 8%, <1% and 1%, respectively (Figure 6). The absence of phagocytosis of zymosan particles
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opsonized with the properdin- and factor D-deficient sera in the presence of these F(ab)_2 suggests that this can only be the effect of the alternative pathway activation and thus, that we are still able to measure the contribution of the alternative pathway complement activation at 3% [vol/vol] serum. At 1%, the serum concentration was too low to obtain any opsonizing effect measurable with phagocytosis by human neutrophils (Figure 4B).

Control experiments

We confirmed the involvement of C2 in MBL-mediated opsonization of zymosan with a C2-deficient serum. Phagocytosis of zymosan particles opsonized with C2- and MBL- deficient serum was strongly reduced (9 ± 3%) (Figure 4C) and addition of purified MBL (5 µg/ml, final concentration) did not influence opsonization in the absence of C2 (not shown). However, addition of both MBL and C2 (3 µg/ml, final concentration) restored opsonization to 74% phagocytosis (mean ± 8%) of that observed with the positive control serum. Restoration of opsonization with C2 and MBL was also achieved after blocking the classical pathway with αC1q (89 ± 9% phagocytosis). These results indicate that the classical, Ig-mediated pathway of complement activation is not needed for adequate opsonization of zymosan under our conditions.

Addition of purified MBL, factor D, factor C2 or properdin to MBL-sufficient sera did not enhance the opsonic activity, suggesting that the concentration of these factors in normal serum is not the limiting factor in the opsonization of zymosan, under the conditions used in our experiments (data not shown).

In a series of experiments we used purified L-ficolin [a generous gift from Dr Matsushita, Fukushima Medical University, Fukushima, Japan] in a concentration of 2 µg/ml. Western blotting with anti-ficolin G4 showed normal product. Moreover, in an ELISA for L-ficolin with anti-ficolin G5, we found serum concentration of 5.3-7.4 µg/ml, 95% CI, among 34 samples, some of whom were MBL-deficient (data not shown). The opsonophagocytosis assay applied seems relatively ficolin-independent, because we found very low phagocytosis of zymosan after blocking with αC1q and αMBL during opsonization with normal sera containing normal levels of ficolin.

Discussion

In this study we have shown that the alternative pathway amplification loop enhances the MBL-mediated route of complement activation for optimal opsonization of zymosan. The opsonophagocytosis assay and the complement deficient sera that were used in these experiments allowed us to measure accurate and strictly MBL-dependent opsonization, by blocking the classical pathway with αC1q F(ab)_2 fragments. Although the opsonizing activity of other serum complement components cannot be fully excluded, the remaining opsonizing activity in 3% MBL-deficient serum with αC1q is most likely due to alternative pathway activation. Opsonization and
MBL-mediated opsonization enhanced by alternative pathway

subsequent phagocytosis were restored upon MBL addition in vitro. Our results indicate that factor D as well as properdin is essential for MBL-mediated opsonization and subsequent phagocytosis of zymosan by human neutrophils. It thus appears that not only the classical pathway (C4- and C2-mediated) of complement activation but also the amplification loop of the alternative complement route is required for adequate complement-mediated opsonization of zymosan by MBL.

It is known that MBL-mediated complement activation is initiated after binding of MBL to sugar structures on the microbial surface. In this study zymosan was used as test particle because zymosan opsonization is highly dependent on MBL binding. MBL is found in serum complexed with the MASP-s and a small 19 kDa protein (MAP19). MASP2 activates complement by cleaving C4 and C2 (33). The MASP2-mediated cleavage results in the generation of covalently bound C4b2a complexes, which are able to function as C3 convertase enzymes resulting in C3 cleavage and the production of opsonic C3b/iC3b fragments. The involvement of C2 in complement activation via the lectin pathway was confirmed in our study with C2-deficient serum.

Apart from MBL, two serum ficolins, L-ficolin and H-ficolin, have been identified to bind MASP-s. The ficolins are also similar in quaternary structure to C1q and MBL, and may thus result in complement activation as an alternative lectin route. Only L-ficolin activates a complement activation route that leads to opsonization (34). The third ficolin, the non-serum M-ficolin, is known to be secreted by porcine and human neutrophils (35, 36). However, purified serum L-ficolin did not completely restore phagocytosis of zymosan when added up to 5 µg/ml final concentration to MBL-deficient serum depleted from anti-zymosan specific antibodies [data not shown]. This is above the normal median ficolin concentration of 1.1-12.8 µg/ml (median 3.7) ([37] and unpublished results). Thus, L-ficolin, and very likely also H-ficolin and M-ficolin, do not appear to play an important functional role in zymosan opsonization under the conditions used in our experiments.

Zymosan is known to stimulate Toll-like Receptor 2 (TLR2) expressing cells (PMN) (38, 39). To exclude TLR2 involvement in our experiments, we have tested the uptake of unopsonized zymosan particles up to 90 minutes by PMN under resting experimental conditions (phagocytosis <1%) and after pre-activation with Platelet-Activating Factor (PAF; 10 ng/ml) (phagocytosis 2.5 ± 0.2%) or Tumor Necrosis Factor-α (TNFα; 10 ng/ml) (phagocytosis 2.4 ± 0.3%). Uptake was compared to MBL-sufficient control serum set at 100% (data not shown). We conclude that there is no phagocytosis of zymosan particles via direct interaction with the TLR2 on unprimed or preactivated PMN in our assay.

The alternative complement pathway is activated by a mechanism different from the classical and lectin pathways. Once formed, C3bBb turns over more C3, thus producing strong opsonization. With regard to the involvement of the alternative complement cascade in the lectin route, we performed experiments with factor D- and properdin-deficient sera. Factor D cleaves its
substrate factor B to form the C3b–factor B complex. There is no endogenous inhibitor of factor D which circulates in an active form that does not require proteolytic activation. Properdin is present in serum as an enhancer of the alternative complement pathway. It is of central importance to the deposition of the activated complement fragment C3b on the surfaces of pathogens by preventing the dissociation of the Bb catalytic subunit from the inherently labile C3bBb complexes.

Our experiments with factor D-deficient sera revealed decreased phagocytosis compared to phagocytosis of zymosan opsonized with a positive control serum. Under these conditions, the remaining phagocytosis measured was supposed to be mediated via opsonization by specific zymosan antibodies and the classical pathway of complement activation. Therefore, we blocked the classical pathway by addition of αC1q. The opsonization was only restored to normal levels by the combined addition of factor D and MBL, suggesting that the amplification loop of the alternative pathway is required for optimal MBL-mediated opsonization, even at low serum concentrations. Experiments with properdin-deficient serum showed similar results.

We performed zymosan opsonization at 3% serum concentration to obtain optimal effect of the lectin pathway and sensitive discrimination between MBL-deficient and MBL-sufficient sera. Experiments at different serum concentrations (1-10% [vol/vol] serum) demonstrated that, even although in 10% serum the alternative pathway activation is more prominent, at 3% serum there is still phagocytosis of zymosan opsonized with control serum in the presence of antibodies that block the lectin pathway (αMBL) and the classical pathway (αC1q). At the same time, in the factor D- and properdin-deficient sera, no remaining opsonization was observed at these serum concentrations under these blocking conditions. These data suggest that the alternative pathway activation is not limited to high serum concentrations but may well contribute to opsonization at lower serum concentrations, up to 3% [vol/vol].

The role of the alternative pathway in MBL-mediated complement activation found in this study and the compensating role of the antibody-mediated classical pathway activation for impaired opsonization in MBL-deficient individuals found by Roos et. al (16) suggests that the complement system does not consist of independently operating pathways but is a highly interacting and complex system. The amplification loop plays an enhancing role in the opsonization of zymosan via the lectin pathway, supporting the increased risk of infections in MBL-deficient individuals.

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MBL-mediated opsonization enhanced by alternative pathway

References

Chapter 2


