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

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Citation for published version (APA):

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Mannose-binding lectin facilitates opsonophagocytosis of yeasts but not of bacteria, despite MBL binding

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*J. Immunology* 2008 March; 180 (6): 4124-4133
Abstract

Mannose-binding lectin (MBL) is a serum protein of the innate immune system. After binding to a micro-organism, MBL in complex with MBL-associated serine proteases (MASPs) activates the complement system, resulting in cleavage of complement factor C3. Cleaved C3 on the surface of the micro-organism mediates opsonization for clearance, but the impact of MBL on subsequent phagocytosis has not been widely studied.

We investigated the role of MBL in complement activation and phagocytosis of various bacteria and yeast species by flow cytometry. We measured both the C3 deposition during serum opsonization of fluorescent labeled micro-organisms as well as subsequent uptake of these micro-organisms by human neutrophils. In MBL-deficient sera a consistently decreased C3 deposition on both zymosan and Candida albicans was found, and a reduced phagocytosis by neutrophils that was restored by exogenous MBL. This indicates that the lectin pathway of complement activation is important for the opsonophagocytosis of yeasts. In contrast, the C1q-dependent classical pathway dominated in the opsonization and phagocytosis of Staphylococcus aureus, Streptococcus pneumoniae and Escherichia coli, whereas no effect of MBL was found. Both the lectin and the classical pathway of complement activation were highly amplified by the alternative route for opsonophagocytosis by neutrophils of yeast as well as microbial species.

In summary, our data demonstrate that yeast species are preferentially opsonized and subsequently phagocytized via activation of the lectin pathway of complement, whereas the uptake of bacterial strains was found to be largely MBL independent.

Introduction

The innate immune system, including the complement system acts as a first line of defense against pathogens. There are three pathways of complement activation, the classical, the alternative and the lectin pathway. Each pathway induces cleavage of complement factor C3, forming C3b and iC3b that bind to micro-organisms and act as opsonins for the clearance of pathogens. The lectin pathway of complement activation is based on the recognition of pathogen-associated molecular patterns, such as mannose, peptidoglycans and lipopolysaccharide. These sugars are recognized by ficolins and collectins, which are initiators the lectin pathway of complement activation (1). One of the collectins is mannose-binding lectin (MBL).

MBL is an oligomeric molecule that is synthesized in the liver. In human serum MBL oligomers, from dimers to hexamers, are present. The MBL2 gene encoding MBL on chromosome 10q21 contains several single nucleotide polymorphisms (SNPs). Three independent coding SNPs in exon 1 (variant alleles B, C, D, wild-type denominated as A) disrupt the collagenous structure
of the protein and hampers the formation of S-S bridges between subunits, leading to small, non-functional MBL molecules (2, 3). In addition, three SNPs in the promoter region have a regulatory influence on the serum MBL concentration. These SNPs are denominated H/L, X/Y and P/Q. The six SNPs together result in seven generally known haplotypes: HYPA, LYPA, LYQA with normal MBL concentrations, and LXPA, LYPB, LYQC and HYPD with reduced or very low MBL concentrations (2), leading to MBL deficiency. The prevalence of MBL deficiency in a Caucasian population is about 30% (1, 4). MBL deficiency has been defined in various ways, both at the plasma level and by corresponding genetic haplotypes (3, 5). Previously, we have defined MBL deficiency in a Caucasian population as MBL concentrations below 0.7 μg/ml (6). Most individuals lacking MBL do not suffer from adverse consequences. Therefore, it seems likely that for MBL deficiency to cause clinical symptoms, it must occur concomitantly with other immune deficiencies, i.e. an impaired mucosal barrier or immune system (cystic fibrosis, newborns and chemotherapy-treated patients).

MBL binds to several clinically relevant pathogens (7, 8). Many patient studies have reported a correlation between MBL deficiency and increased susceptibility to various infection-related diseases (9). A high association was found for MBL insufficiency and increased bacterial infection in patients with neutropenia and meningococcal sepsis. Low MBL levels also appeared to predispose individuals to HIV infection (10).

The ability of MBL to bind with high affinity to mannose and N-acetyl-glucosamine oligosaccharides present on the surface of various Gram-positive and Gram-negative bacteria, fungi and yeast particles has been reported. MBL binds strongly to Candida species, Aspergillus fumigatus, Staphylococcus aureus, and beta-hemolytic group A streptococci. An intermediate binding of MBL has been found for Escherichia coli, Klebsiella species, and Haemophilus influenzae type b. In contrast, beta-hemolytic group B streptococci, Streptococcus pneumoniae, and Staphylococcus epidermidis bind MBL weakly (8).

The complement activation following upon MBL binding to pathogens is dependent on MASP (11),(12). After MASP binding to MBL the complement cascade is activated via the formation of the C4b2a complex, which is able to generate and bind the opsonins C3b and iC3b, thereby facilitating opsonophagocytosis. Complement activation after binding of MBL has mainly been studied after binding of MBL to mannan-coated polystyrene (13, 14), with C3b or C4b formation as read-out. Therefore, the precise contribution of MBL to pathogen opsonization and subsequent uptake by phagocytes has remained unclear. Since MBL is being considered as plasma-derived or recombinant product for therapeutic application, it is important to know what the role of MBL is, in both the early phase of opsonization as well as in the subsequent phagocytosis. We investigated the impact of MBL on phagocytosis of various micro-organisms by human neutrophils. In addition, we studied the extent of complement deposition on various Gram-positive and Gram-negative bacteria as well as on yeast particles in relation to the efficiency of phagocytosis of these pathogens.
Material and Methods

Serum samples

Blood was obtained with informed consent from MBL-sufficient and MBL-deficient healthy volunteers and from patients with a complement deficiency or X-linked agammaglobulinemia (XLA). Serum aliquots were stored at -80°C until tested. DNA was isolated from white blood cells, by means of the QiAmp blood mini kit (Qiagen, Hilden, Germany). The activities of the classical (CH50) and alternative (AP50) pathways of the complement system were measured as described (15).

MBL serum concentration and MBL2 genotype

To select MBL-sufficient and MBL-deficient donors, MBL serum concentrations were measured by an ELISA technique as previously described (16). Briefly, mannan was coated to a solid phase and incubated with sera. Thereafter, biotinylated mouse-anti-MBL-1, (10 μg/ml, Sanquin, Amsterdam, the Netherlands) was used as detection antibody. Genotyping of the three generally known promoter polymorphisms and three exon-1 point mutations was performed with a Taqman assay with specific primers and minor groove binding (MGB) probes for each point mutation (16). Donors with MBL serum concentrations ≤0.05 μg/ml and an O/O or XA/O genotype were selected to obtain profoundly MBL-deficient sera.

Polymorphonuclear (PMN) leukocyte isolation

Fresh isolates of PMN were purified from whole blood by centrifugation over a Percoll gradient as described by Roos and De Boer (17). After lysis of the erythrocytes, the PMN were washed in Phosphate-Buffered Saline (PBS, pH 7.4, NPBI International, Emmer-Compascuum, The Netherlands) and adjusted to 10^7 cells/ml in HEPES medium (132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM HEPES (Sigma, Steinheim, Germany), 2 mM CaCl₂, 5.5 mM glucose and 0.5% (w/v) human serum albumin (Sanquin), pH 7.4). All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise indicated.

Preparation of antibody F(ab')₂ fragments

F(ab')₂ fragments were made from a blocking monoclonal mouse antibody against C1q (anti-C1q-85; Sanquin (18, 19)). One mg of antibody was dialyzed against 0.1 M tri-sodiumcitrate-dihydrate, pH 3.5. Pepsin (Sigma) was added to a concentration of 25 μg/ml, and samples were incubated overnight at 37°C. The F(ab')₂ fragments were dialyzed against PBS.

Culture and FITC-labeling of pathogens

Zymosan, prepared from the yeast Saccharomyces cerevisiae (lot no. 1389F, MP Biochemicals, Solon, OH, USA) was suspended in PBS at a concentration of 10 mg/ml. Candida albicans, Staphylococcus aureus 502A and Escherichia coli O54 were cultured in LB medium.
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(Luria Broth, Becton Dickinson, Sparks, MD, USA) and Streptococcus pneumoniae serotype 3 on low oxygen in BHI (Brain Heart Infusion; Invitrogen, Paisley, Schotland). All micro-organisms were grown at 37°C until an OD₆₀₀ of 1 (log phase) had been reached. After washing with PBS, all pathogens were resuspended in PBS at 1.0 x 10⁹ CFU/ml. All suspensions were labeled for 30 minutes at 37°C with 10 μg/ml of FITC (Sigma) in the dark. Labeling was followed by washing 3 times with PBS, after which the pathogens were resuspended in PBS to the starting concentration. Aliquots of the FITC-labeled pathogens were kept in the dark at –30°C.

Opsonization

FITC-labeled organisms (zymosan: 250 μg, pathogens: 7.14 x 10⁶ CFU) were opsonized in 250 μl of 3% (v/v) human serum in HEPES medium, during 30 min of incubation at 37°C while shaking. Thereafter, the opsonized micro-organisms were washed twice and resuspended in 25 μl of HEPES medium. The classical pathway of complement was blocked with F(ab’)_2 fragments of a monoclonal antibody against C1q (20 μg/ml). Complement C3 was inhibited by compstatin 4 (1MeW)⁷W (20), a 13-amino-acid cyclic peptide that binds to the b-chain of C3. The 4(1MeW)⁷W compstatin contains 1-methyltryptophan at position 4, followed by a peptide containing 5-fluorotryptophan at position 7, which increased the inhibitory activity of the peptide. Plasma purified MBL (2 μg/ml, MBL-SSI, Statens Serum Institute, Copenhagen, Denmark), C1q (100 μg/ml, Sanquin), and complement factor D (1 μg/ml; Quidel, San Diego, CA, USA) were used to exclude direct opsonization through these proteins and to restore deficient sera during opsonization.

Phagocytosis assay

Phagocytosis was determined by flow cytometry as described previously (6), with some minor adjustments for optimal conditions for each micro-organism tested. Opsonized FITC-labeled micro-organisms (25 μl) were mixed with neutrophils (0.5x10⁶) in HEPES medium in a final volume of 250 μl, to start phagocytosis. Incubation was performed shaking, at 37°C. At different time points (0, 2, 5, 10 and 20 minutes) 50-μl samples were taken and added directly to 150 μl of ice-cold stop buffer (20 mM NaF [Sigma], 0.5% paraformaldehyde [Merck], 1% [v/v] bovine serum albumin [Sigma] in PBS), to stop phagocytosis.

Phagocytosis was determined by flow cytometry (FACSCalibur, Becton Dickinson). Green Fluorescence (FL1) of 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 (FITC-positive PMN). Non-phagocytized micro-organisms were excluded from analysis by gate R1.

As a control the complement-independent phagocytosis was determined. Micro-organisms opsonized with heat-inactivated serum were added to PMN that had been blocked with 25 μg of anti-FcgRII (Fab fragments of clone IV.3) (21) per reaction.
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Results of the opsonophagocytosis assay are expressed as percentage of phagocytosis compared to a positive control (pathogens opsonized with MBL-sufficient serum), which was set at 100%. All samples were tested on three different days (n=3), with freshly isolated PMN from healthy donors other than those listed in Table 1. Flow cytometry data were confirmed by fluorescence microscopy (data not shown).

Complement deposition

The binding of several complement components to the pathogens was investigated by FACS analysis (quantitative) and Western blotting (qualitative). Pathogens were opsonized as described above, washed and resuspended in HEPES medium. Opsonization was performed with serum alone, serum in the presence of 2 µg/ml plasma-purified MBL or serum in the presence of 10 µg/ml anti-C1q (anti-C1q-85). In one set of experiments, the micro-organisms were incubated with various concentrations of plasma-purified MBL alone. For FACS analysis, the opsonized pathogens were incubated with monoclonal antibodies either directed against MBL (anti-MBL-1) or against C3 (anti-C3-9, Sanquin) in a final concentration of 20 µg/ml, shaking for 1 hour at 37ºC. After washing, pathogens were resuspended in HEPES medium and incubated with rat-anti-mouse PE (1:100; Dako, Glostrup, Denmark) for 30 minutes at 37ºC, shaking and in the dark. After washing, samples were analyzed by flow cytometry (FACSCalibur). All samples were tested on three different days (n=3). The mean fluorescence intensity (MFI) of the PE signal, representing the C3 deposition, is expressed as relative C3 deposition compared to donor 1, which was set at 100 AU (mean ± SEM).

For Western blotting, samples were washed and resuspended in HEPES medium without albumin and boiled at 95ºC for 5 minutes in sample buffer (125 mM Tris, 20 % (v/v) glycerol, 5% (w/v) SDS and 0.02% (w/v) Coomassie blue) without β-mercapto-ethanol or dithiotreitol. SDS-PAGE was performed on 5% acrylamide gels with Precision Plus Protein™ standard (Biorad, Hercules, CA, USA) as molecular marker. Proteins from the SDS-PAGE gel were transferred to a polyvinylidene difluoride membrane (Biorad). Membranes were blocked with milk/TBST (5% (w/v) milk (Campina, Eindhoven, the Netherlands), 10 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20, pH 7.4). After washing, the membranes were incubated in milk/TBST containing the primary monoclonal antibody (1:1000) anti-iC3b (Quidel), anti-MBL-6 (Sanquin) or anti-L-ficolin (GN5, Cell Sciences, Uden, the Netherlands). After washing, the membranes were transferred to a 1:2500 secondary antibody dilution (goat-anti-mouse, GE Healthcare, Amersham, UK) in milk/TBST. Before detection with ECL Western blot reagent kit (Pierce, Rockford, IL, USA) membranes were washed thoroughly with TBST and PBS. Silver staining with Silver Quest staining kit (Invitrogen) was performed as loading control.

Statistics

Data from the opsonophagocytosis and complement deposition assays are presented as mean
MBL binding facilitates opsonophagocytosis of yeast

± S.E.M. Results were compared with an unpaired $t$-test or one-way analysis of variance (ANOVA) when applicable. The Bonferroni post-hoc test was used for multiple comparisons. Differences were considered to be significant when $p$-values were $<0.05$. SPSS 14 and Prism 4 computer software were used for analysis.

Results

**MBL characteristics of the sera**

From the sera of 100 healthy donors we randomly selected 6 MBL-sufficient sera (serum 1-6) and 6 MBL-deficient sera (serum 7-12). Sera from donors 13-16 had other complement deficiencies and were used as controls. The MBL concentration and genotype (wildtype A/A, heterozygous MBL-deficient A/O or homozygous MBL-deficient O/O) of these donors are listed in Table I. All sera had sufficient levels of MASP-2, C4 and C3, except the C3 nephritic factor serum (C3Ne; data not shown).

**Opsonophagocytosis of different pathogens**

MBL-dependent opsonization was performed with 3% (v/v) serum for 30 minutes at 37°C for all pathogens. These opsonization conditions were chosen to obtain optimal discrimination between MBL-sufficient and MBL-deficient sera (6). Phagocytosis of the opsonized pathogens was followed up to 20 minutes, when phagocytosis had reached a plateau phase. Opsonophagocytosis was C3-mediated in all micro-organisms tested, since 56°C heat-inactivated serum or purified MBL, C1q or factor D alone did not opsonize the micro-organisms. Activation of complement via direct binding of MBL to antibodies was excluded with the use of an XLA serum, which gave similar results as MBL-sufficient sera in the presence of anti-C1q antibodies.

Phagocytosis of zymosan opsonized with MBL-sufficient sera was 141 ± 26% compared to phagocytosis of zymosan opsonized with a well-characterized MBL-sufficient serum (serum 1 from Table I was set at 100%; see also (6)). The opsonophagocytosis of zymosan with MBL-deficient sera was 39 ± 3% (Figure 1A). The contribution of the lectin pathway activation for phagocytosis of zymosan was statistically significant ($p<0.01$). After blocking the classical pathway with an inhibiting monoclonal antibody directed against C1q, the difference in opsonophagocytosis with MBL-sufficient and MBL-deficient sera was even stronger. The anti-C1q antibody was tested in a Wielisa (Wieslab, Lund, Sweden) and found to specifically block the classical pathway of complement activation without affecting the alternative or lectin pathway (data not shown). All $p$-values of the opsonophagocytosis data in Figure 1 are summarized in Table II.

The lectin pathway of complement activation was also significantly involved in the opsonophagocytosis of *C. albicans*. The average phagocytosis of *C. albicans* opsonized with MBL
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Table I. Donor description. Indicated are sex of the donor, MBL concentration, MBL2 exon 1 mutation (A/A = wildtype, A/O = heterozygous mutation, O/O = homozygous mutation) and description of other deficiencies when applicable.

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<th>other deficiencies</th>
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<td>0.8</td>
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Table II. Statistics of Figure 1.

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<td>MBL-</td>
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MBL binding facilitates opsonophagocytosis of yeast -sufficient sera was $108 \pm 7\%$, whereas the opsonophagocytosis with MBL-deficient sera was $72 \pm 8\%$ (Figure 1B). Inhibition of the classical pathway resulted in reduced phagocytosis of *C. albicans* opsonized with MBL-sufficient or MBL-deficient sera, respectively.

In contrast to the zymosan and *C. albicans*, the same MBL-sufficient and MBL-deficient sera did not show a difference in opsonophagocytosis of the various bacterial strains tested (Figure 1C-1E), indicating that the lectin pathway of complement activation did not contribute to a large extent to the opsonophagocytosis of these bacteria. However, inhibition of the classical pathway of complement activation during opsonization with either MBL-sufficient or MBL-deficient sera induced a 2-3 fold reduction in the subsequent phagocytosis of *S. aureus*. This implicates a strong role for the C1q-dependent classical pathway of complement activation in the opsonization of this strain of *S. aureus*.

The findings regarding opsonophagocytosis of *S. pneumoniae* serotype 3 (Figure 1D) and *E. coli* (Figure 1E) were almost similar to those obtained with *S. aureus* bacteria. No difference in phagocytosis was noted between opsonization with MBL-sufficient or MBL-deficient sera, and again a statistically significant reduction was found upon inhibition of the classical pathway by anti-C1q.

**MBL and C3 deposition on the various pathogens**

The difference between the mainly C1q-mediated opsonophagocytosis of the bacteria and the highly MBL-dependent opsonophagocytosis of zymosan and *C. albicans* might

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**Figure 1.** Phagocytosis by human neutrophils of different pathogens. (A) Zymosan, (B) *Candida albicans*, (C) *Staphylococcus aureus*, (D) *Streptococcus pneumoniae* and (E) *Escherichia coli*. Each pathogen was opsonized with MBL-sufficient sera (MBL+), MBL-deficient sera (MBL-), MBL-sufficient sera with MoAb anti-C1q (MBL+/bC1q) or MBL-deficient sera with MoAb antiC1q (MBL-/aC1q). The bars in each graph represent the average opsonophagocytosis (± SEM) of 6 donors, measured on three different days. Significant differences between opsonization with MBL-sufficient and -deficient donors is presented as * (p<0.05) and ** (p<0.005). All statistics of this figure can be found in Table II.
be caused by differences in ligand availability for MBL. Binding of MBL to the various microorganisms was therefore determined (Figure 2). MBL binding to all microorganisms appeared to be dose dependent until a certain plateau was reached. Zymosan and *C. albicans* showed maximal MBL binding at higher MBL concentrations than did *S. pneumoniae* and *S. aureus*, while *E. coli* bound only little MBL.

We next investigated whether complement C3 activation on the various microorganisms was dependent on MBL binding. A frequently used method to measure classical or lectin pathway activation of complement is the activation of C3 and subsequent deposition of immobilized C3 fragments upon binding of MBL or C1q in solid-phase ELISA systems. Instead of using ELISAs, we determined the C3 binding by flow cytometry on the different pathogens during opsonization, using the same conditions as in the opsonophagocytosis assay.

First, a titration up to 10% (v/v) serum was performed with MBL-sufficient and MBL-deficient sera. As shown for zymosan (Figure 3A) and *S. aureus* (Figure 3B) as representative examples for the particles used, there is hardly any complement deposition at 1% serum, whereas 95% of the zymosan and 72% of the *S. aureus*, respectively, were positive for C3 when opsonized at 3% MBL-sufficient serum. With MBL-deficient sera, on average 37% of the zymosan and 83% of the *S. aureus* were found C3-positive.

We did not find any difference in the extent of C3 deposition on *S. aureus*, expressed as mean fluorescence intensity (MFI), comparing MBL-sufficient and MBL-deficient sera. This was also true for the other bacteria tested. In contrast, with zymosan, both the percentage of C3-positive particles as well as the extent of C3 deposition per particle were decreased in MBL-deficient serum (Figure 3A, middle and lower panels). All particles were C3 positive at 3% MBL-sufficient serum used during opsonization, although C3 deposition per particle still increased at 5 or 10% serum (Figure 3A, lower panel).

We chose to use 3% serum for opsonization in all further experiments. Figure 4A shows that C3 binding to zymosan opsonized with either MBL-sufficient or MBL-deficient sera differed significantly (p=0.01), which became even more prominent when the classical pathway was blocked with anti-C1q (p=0.005). This finding corresponded with the results from our
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Phagocytosis assay. Addition of plasma-derived MBL (22) resulted in increased C3 deposition (p=0.005) and a disappearance of the difference between the MBL-sufficient and MBL-deficient sera in C3 deposition (Figure 4A). Addition of anti-C1q only decreased the C3 deposition on zymosan in the presence of MBL-deficient sera, indicating that in the absence of MBL some C1q-dependent C3 activation takes place on the zymosan surface.

Similar to zymosan, the MBL-deficient sera also showed less C3 deposition on C. albicans compared with the MBL-sufficient sera, in particular when anti-C1q was present during opsonization (p=0.002) (Figure 4B). These results indicate that C. albicans is opsonized via both the classical and the lectin pathway of complement activation. Addition of MBL resulted in increased C3 deposition on C. albicans with MBL-sufficient or MBL-deficient sera (p<0.005).

In contrast to the observations with yeast particles, the C3 deposition on all bacterial pathogens tested seemed to be MBL independent. Neither S. aureus nor S. pneumoniae showed any difference in C3 deposition after opsonization with MBL-sufficient or MBL-deficient sera (Figure 4C and 4D). Addition of plasma-derived MBL also did not raise C3 deposition on any of the bacteria. Addition of blocking anti-C1q strongly decreased the C3 deposition in all cases (p<0.0001, Figure 4C for S. aureus and p<0.001, Figure 4D for S. pneumoniae). Together with the phagocytosis data these results strongly suggest that MBL does not have a significant role in opsonization of S. aureus or S. pneumoniae.
The enhancing role of the alternative pathway amplification loop.

Three alternative pathway-deficient sera, two from different homozygous factor D-deficient donors and one from a properdin (factor P)-deficient donor, were used to analyze the role of the alternative pathway in the opsonization of the different pathogens. All three alternative pathway-deficient sera had low MBL concentrations as well (Table I). As controls we used C3Ne serum devoid of any C3, the potent C3-blocking agent compstatin 4(1MeW)7W (20), and serum in which complement has been inactivated by treatment for 30 minutes at 56°C (Figure 5, and data not shown).

Opsonophagocytosis of zymosan was clearly reduced when the MBL-sufficient control (set at 100%) was compared with the average of the 3 sera with a combined MBL and alternative pathway deficiency (Figure 5, AP-def, 17.4 ± 3.9% phagocytosis). MBL-deficient sera resulted in an opsonophagocytosis of about 40% (Figure 1A). Together, this confirms our earlier data (6) on the enhancing role for the amplification loop of the alternative pathway in the opsonization process. Complement dependency was indicated by the lack of phagocytosis after opsonization with either heat-inactivated serum, C3Ne serum (Figure 5A), or with control serum to which compstatin had been added.

C. albicans showed a complement-independent phagocytosis component, with 16-35% phagocytosis after opsonization with C3Ne serum or control serum with compstatin or heat-inactivated control serum (Figure 5B). The average opsonophagocytosis with the combined MBL/alternative pathway-deficient sera was 45%, again significantly (p<0.0005) lower

**Figure 4.** C3b deposition on different pathogens. (A) Zymosan, (B) Candida albicans, (C) Staphylococcus aureus, and (D) Streptococcus pneumoniae. Each pathogen was opsonized with 6 MBL-sufficient (●) and 6 MBL-deficient (■) sera. From left to right, with sera alone, with sera to which 2 mg/ml plasma-purified MBL had been added and with sera in which the classical pathway had been blocked. The bars in each graph represent the average (± SEM) C3 deposition in AU of the 6 sera, measured on three different days. Significant differences between opsonization with MBL-sufficient and -deficient sera are presented as * (p<0.05) and ** (p<0.005).
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**Figure 5.** C3 deposition (left) and phagocytosis (right) after opsonization with several control sera, showing the complement dependency of the phagocytosis and the enhancing role of the alternative pathway amplification loop. (A) Zymosan; (B) Candida albicans; (C) Staphylococcus aureus; and (D) Streptococcus pneumoniae. The left panel shows the average (± SEM) C3 deposition in AU, measured on three different days, the right panel shows the average (± SEM) phagocytosis in % (compared to opsonization with 3% (v/v) MBL-sufficient serum), measured on three different days. In each graph the microorganisms were opsonized, from left to right, with PBS (unopsonized), MBL-sufficient serum, alternative pathway (2 x factor D and 1x properdin)-deficient (AP-def) sera (n=3), C3-deficient serum (C3Ne, C3 concentration <0.05 g/L), alternative pathway-deficient sera in the presence of blocking MoAb anti-C1q (n=3), MBL-sufficient serum to which the complement C3-inhibitor compstatin 4(1MeW)7W had been added, and MBL-sufficient serum heat-inactivated for 30 minutes at 56°C. The dashed line represents the background from the non-opsonized control.
than the 72% phagocytosis observed after opsonization with MBL-deficient sera.

Although MBL-sufficient sera as well as MBL-deficient sera opsonized *S. aureus* (Figure 5C) and *S. pneumoniae* (Figure 5D), both micro-organisms were less efficiently phagocytized after opsonization with the alternative pathway-deficient sera (70 and 64%, respectively). Thus, also for bacteria the amplification loop is of importance for adequate opsonization. Although the bacteria opsonized with the C3Ne serum, with the control serum in the presence of compstatin or with heat-inactivated control serum were slightly better phagocytized than the unopsonized bacteria, the major part of the opsonization was complement dependent.

These data are supported by the reduced C3 deposition found after opsonization with the alternative pathway-deficient sera or the C3Ne serum (left panel in Figure 5 and Figure 6). Only the C3 deposition on *S. aureus* after opsonization with the combined alternative pathway- and MBL-deficient sera was still high, suggesting that opsonization of *S. aureus* was mediated predominantly by the classical pathway of complement activation. The remainder of the phagocytosis observed with *C. albicans* and *S. pneumoniae* in the absence of active C3 was blocked by an antibody directed against the IgG receptor, FcgRII (CD32, Fab fragments of clone IV.3, data not shown).

![Figure 6. Western blot of non-reduced gels stained top to bottom, for MBL, iC3b, L-ficolin and silver staining of the gel. Samples from left to right: 1-4, sera from donor 1 (MBL+); donor 7 (MBL-); donor 13 (D-deficient); and donor 16 (C3Ne); using these sera, eluates of opsonized zymosan, Candida albicans, Staphylococcus aureus and Streptococcus pneumoniae were compared. All micro-organisms were opsonized with sera samples 1-4 in the same order. As loading control a silver staining was performed, with the 60-kDa band as representative for the whole lane.](image)
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To further elucidate the binding of complement proteins to the surface of the various microorganisms, Western blots were prepared. Sera from donor 1 (MBL A/A), donor 7 (MBL O/O), donor 13 (factor D-deficient / MBL A/O) and donor 16 (C3Ne-positive / MBL A/A) and eluates of zymosan, C. albicans, S. aureus and S. pneumoniae opsonized with these sera, were analyzed for MBL-, C3 fragments and L-ficolin binding. Figure 6 shows that mainly the higher oligomer (tetramer to hexamer) MBL bound to the micro-organisms, although the dimer and trimer were present in the serum as well. Zymosan and C. albicans showed more MBL binding than did the two bacterial strains. MBL O/O or A/O genotypes (sera 2 and 3) showed a low level of MBL in the serum and no high-oligomer MBL bound to the micro-organisms. L-ficolin was equally present in all sera, but only little binding to the micro-organisms was detected. Although some L-ficolin binding to S. aureus and S. pneumoniae was detected, it was only a small fraction of the total L-ficolin present in the sera.

The detection of C3 fragment binding by Western blot was too sensitive and hence could not be used to discriminate between MBL-sufficient and MBL-deficient sera. On the other hand, it clearly showed that the alternative pathway amplification loop is of great importance for C3 deposition on all micro-organisms tested. Two distinct bands of iC3b are visible on the Western blot, because C3 bound covalently to the micro-organisms ran at a higher position than the C3 in serum.

Discussion

Our study focused on opsonization and subsequent phagocytosis of various micro-organisms by neutrophils and resulted in several important observations. First, the deposition of C3 as a critical component of the various complement activation cascades did not always correlate with the extent of phagocytosis by neutrophils. Once a certain threshold has been reached, neutrophils did not show a more efficient uptake, even when more C3 was attached to the micro-organisms. Secondly, when MBL is considered separately, we found, in contrast to bacteria, that MBL-deficient sera consistently mediate a decreased C3 deposition on both zymosan and Candida albicans, and as a consequence a significantly reduced phagocytosis by neutrophils. We therefore conclude that yeast species are preferentially opsonized via activation of the lectin pathway of complement. In contrast, even though MBL was detected on the surface of the bacteria, we did not observe any significant contribution of MBL to opsonophagocytosis of these micro-organisms. C1q-mediated classical pathway activation dominated in the bacterial opsonophagocytosis response. Finally, our findings indicate that both the classical and the lectin pathway of complement activation are highly amplified by the alternative pathway for the effector function of C3-dependent phagocytosis by neutrophils.

There is a vast and diverse body of literature about MBL binding to micro-organisms or
MBL-mediated complement activation after binding to a mannan-coated polystyrene surface, but information is lacking on the role of MBL in promoting phagocytosis. For instance, for \textit{S. aureus} several publications have reported a role for MBL in the C3 deposition on these bacteria but nothing about the uptake of the opsonized \textit{S. aureus} by human neutrophils. Krarup \textit{et al.} (23) reported that MBL does not bind to encapsulated \textit{S. aureus}. On the other hand, Neth \textit{et al.} (24) found an increase in opsonophagocytosis of \textit{S. aureus} after addition of exogenous MBL-MASP to MBL-deficient serum. Furthermore, Lynch \textit{et al.} (25) demonstrated complement activation upon \textit{L}-ficolin binding to \textit{S. aureus}. In an MBL-knockout mouse model infection with \textit{S. aureus} CP5 resulted in decreased C4b deposition and greater mortality (26). However, the clinical relevance for human disease is unclear. In our hands, MBL did not significantly contribute to phagocytosis of \textit{S. aureus}.

Although combined data from the clinical studies of Roy \textit{et al.} (27), Kronborg \textit{et al.} (28) and Moens \textit{et al.} (29) gave a small but significantly increased risk of invasive pneumococcal disease in patients with homozygosity for MBL variant alleles, we did not find an important \textit{in vitro} contribution of MBL in our opsonophagocytosis assay with a common serotype of \textit{S. pneumoniae}. MBL was reported earlier not to bind to encapsulated \textit{S. pneumoniae} (23), but we detected high-molecular weight bands of MBL in the eluates of the micro-organism after opsonization \textit{in vitro}. Kronborg \textit{et al.} already stated that in several MBL-related disease-association studies the MBL-deficient phenotype is only modifying the outcome of a disease in patients with a concomitant disease or disturbance in the immune system, which renders the interpretation of clinical studies on invasive pneumococcal disease (caused by a wide variety of serotypes) difficult.

The same may hold true for \textit{E. coli} infections. Irrespective of the possible existence of an MBL-dependent C2 bypass mechanism for alternative pathway-mediated C3 activation by \textit{E. coli}-derived endotoxin (30), we did not find a significant involvement of MBL to “whole-cell” \textit{E. coli} opsonophagocytosis. This is in agreement with the findings of Proulx \textit{et al.} (31), and the previously described low MBL-binding capacity of \textit{E. coli} (8).

We analyzed MBL binding to the various micro-organisms in two ways, quantitatively with the flow cytometer and qualitatively by Western blot. The Western blot showed that only the higher oligomeric forms (trimers and higher) bound to the micro-organisms. With the flow cytometer, we found a dose-dependent MBL binding to all micro-organisms and, in the presence of high concentrations of MBL, more binding to zymosan and \textit{C. albicans} than to the three bacterial strains. This might be due to variation in MBL-binding epitopes on the various micro-organisms, but could also be due to differences in surface area of the micro-organisms. The higher MBL binding capacity of zymosan and \textit{C. albicans} may contribute to the higher impact of MBL on opsonophagocytosis of the yeast particles compared to the bacterial strains. However, an essential finding from our studies is the fact that, even though MBL did bind to the bacterial species, it had no influence on the uptake of the bacteria by human neutrophils.
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Our data strongly suggest that, although MBL binds to bacteria, the lectin pathway of complement activation has only a minor role in opsonophagocytosis of the bacteria tested. In contrast, we found a strong role for MBL in the opsonization of yeast particles and their subsequent phagocytosis by human neutrophils. In vitro addition of exogenous MBL (5-fold excess) increased C3 deposition on yeasts as well as their uptake by phagocytes. Our results are in agreement with the binding of MBL to *C. albicans* described previously (32, 33), also reporting an MBL-dependent enhancement of C3 deposition and decrease of disseminating candidiasis in mice upon MBL administration. MBL replacement therapy as appropriate (adjuvant) treatment has been suggested for patients with MBL deficiency and disseminated candidiasis.

Besides MBL, ficolins might also activate the lectin pathway of complement. L-ficolin is the most intensively studied ficolin. Several papers describe the binding of L-ficolin to various capsulated *S. aureus* serotypes, capsulated group B streptococci, and capsulated *S. pneumoniae*, but not to noncapsulated strains (23, 34). Upon binding to the capsulated group B streptococci, L-ficolin/MASP complex-dependent C4 consumption was observed. However, no correlation between the amount of C4 consumption and the amount of L-ficolin binding was found. In our experimental setup, the contribution of ficolins to opsonophagocytosis of the various microorganisms seems to be minimal. Although some L-ficolin binding to *S. aureus* and *S. pneumoniae* was detected, it was only a small fraction of the total L-ficolin present in serum. The opsonophagocytosis of *S. aureus* and *S. pneumoniae* in the MBL-deficient sera with blocking anti-C1q antibodies might be due to L-ficolin opsonization. However, equal phagocytosis was observed after opsonization in the presence of C3-blocking compstatin and after opsonization with alternative pathway/MBL-deficient sera in the presence of inhibiting anti-C1q antibodies (but with intact L-ficolin-mediated lectin pathway activation). This suggests that the contribution of L-ficolin to opsonophagocytosis of *S. aureus* and *S. pneumoniae* is very limited.

Besides complement-mediated opsonization, *C. albicans* and *S. pneumoniae* demonstrated that a complement-independent serum factor contributed to phagocytosis of these species in the presence of complement-deficient serum. Blocking of FcgRII revealed that direct binding of opsonizing antibodies to the microorganisms accounted for about 10-20% of the phagocytosis in case of *C. albicans* and *S. pneumoniae*, corresponding with the data for these microorganisms when complement C3-mediated opsonization was blocked.

Our findings indicate that, in patient studies looking at the effect of MBL deficiency on infection and infection parameters, a distinction ought to be made between bacterial and yeast infections. Our data are of importance for the clinical interpretation of MBL deficiency in patients suffering from recurrent or debilitating infections. In case of yeast infection, MBL substitution therapy, as an adjuvant therapeutic measure, might be beneficial. MBL could also be administered as a preventive measure when patients are at high risk, such as at the neonatal intensive care unit or after (hematopoietic) transplantation, and this may hold true for fungal infections in general. On the other hand, our data suggest that the protective value of MBL against (many) bacterial
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pathogens may be limited.

Acknowledgements

The authors thank Professor J.D. Lambris (University of Pennsylvania Medical School, Philadelphia, USA) for providing the compstatin 4(1MeW)7W and Dr. Inga Laursen (Staten Serum Institute, Copenhagen, Denmark) for the plasma-purified MBL. Professor J.C. Jensenius (University of Aarhus, Copenhagen, Denmark) is gratefully acknowledged for measuring MASP-2 concentrations. We thank Shabman Solati, Margreet Hart and Mieke Brouwer from Sanquin, Amsterdam, Netherlands for the monoclonal antibodies against C1q, MBL and C3, respectively, and Dr. A. Tool, Dr. T. van den Berg and R. van Zwieten (Sanquin) for helpful discussions.

References

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