The role of mannose-binding lectin in vitro and in vivo
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Mannose-Binding Lectin substitution trial: recovery of opsonic function lags behind MBL serum levels

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Abstract

Mannose-binding lectin (MBL) deficiency is associated with increased infection risk or more severe disease outcome in immunocompromised individuals. An open, uncontrolled safety and pharmacokinetic MBL-substitution study was conducted in 12 pediatric oncology patients with chemotherapy-induced neutropenia. Twice weekly MBL infusions, with plasma-derived MBL, proved safe, and the desired MBL trough levels above 1.0 μg/ml were reached (median 1.06 μg/ml). In these patients, we tested whether in vivo MBL substitution increased MBL-dependent in vitro complement activation and opsonophagocytosis of zymosan.

Upon MBL substitution, opsonophagocytosis of zymosan by human neutrophils increased significantly from 24% before MBL infusion to 75% directly after MBL infusion (compared to positive control serum set at 100%, p<0.001), and to 45% prior to the second infusion (p<0.025). MBL-mediated complement C3 and C4 activation showed an increase comparable to that of the opsonophagocytosis. Repeated MBL infusions resulted in an increase in opsonophagocytosis over time. During the first 24 hours after MBL substitution, MASP-2 activation as well as alternative pathway complement activation was suboptimal.

This MBL-substitution study demonstrates that twice weekly infusions with plasma-derived MBL increase MBL-mediated C3 and C4 activation and opsonophagocytosis of zymosan, but efficacy in chemotherapy-induced neutropenic patients can be debated. A phase-II/III MBL-substitution study should demonstrate clinical efficacy.

Introduction

Mannose-binding lectin (MBL) is a serum protein that belongs to the collectin family of proteins. MBL molecules are large macromolecules with a bouquet-like structure, similar to that of C1q. Both MBL and C1q are innate immune proteins that can initiate the complement cascade. C1q initiates the classical pathway of complement activation after binding to antibodies, while MBL initiates the lectin pathway of complement activation after binding to repeating sugar structures on micro-organisms (1, 2). The binding of MBL leads to opsonization of the micro-organisms and a more efficient clearance by neutrophils. Around 1970, a defect in the opsonization of baker’s yeast (Saccharomyces cerevisiae) or its major capsular constituent zymosan, was described for the first time (3, 4). In 1989 it was shown that the lack of opsonization in these sera was caused by MBL deficiency (5). Since then, many associations between MBL deficiency and increased susceptibility to infection with various micro-organisms have been published. MBL-deficient pediatric patients show a higher incidence of recurrent infections, mainly in the upper respiratory tract (6, 7). MBL deficiency is associated with a higher susceptibility to HIV (8, 9), reduced life expectancy in cystic fibrosis patients due to more frequent
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and more severe infections (10), and an increased risk of developing sepsis (11, 12). Currently, it is believed that most individuals with MBL deficiency remain healthy, but that it leads to an increased risk to infections in immunocompromised individuals, i.e. neonates lacking adaptive immunity (13, 14), oncology patients receiving chemotherapy (15, 16), or patients suffering from other immune deficiencies. Associations between MBL deficiency and longer episodes of febrile neutropenia in children and more severe infections in adults with cancer have been described (17-19).

MBL levels are largely genetically determined, but can differ up to ten-fold in individuals with identical genotypes for the six known variants (20). Three structural point mutations in exon 1 of the MBL2 gene, at codon 52, 54 and 57 (D, B and C variant, respectively) prevent formation of MBL oligomer complexes and drastically reduce the MBL level. In addition, three promoter polymorphisms (X/Y, H/L and P/Q) exist that influence the MBL plasma level. Due to linkage disequilibrium only seven haplotypes are found, i.e. HYPA, LYP A, LYQA, LXPA, HYPD, LYPB and LYQC (21). Apart from the B, C and D exon 1 variants, the LXPA haplotype is also associated with reduced or deficient MBL levels (22). Very low or undetectable levels (<0.05 μg/ml) are found in individuals with homozygous exon 1 mutations, intermediately reduced levels are seen in individuals with a heterozygous exon 1 mutation or with the LXPA/LXPA haplotype. In general, the remaining individuals with the homozygous wild-type allele (A) show MBL levels above 1.0 μg/ml, up to more than 10 μg/ml.

Previously, we found that in vitro addition of plasma-derived MBL (1.0-5.0 μg/ml) to MBL-deficient control sera completely restored the opsonic function of these sera, as measured by the phagocytosis of opsonized zymosan by human neutrophils (23). Therefore, MBL-deficient patients with increased infection susceptibility might benefit from MBL substitution therapy. MBL was purified from plasma of Danish blood donors by the Statens Serum Institute (SSI) in Copenhagen, Denmark (24, 25). In a phase-I trial, carried out in 20 MBL-deficient but healthy adult volunteers, MBL-SSI substitution appeared to be safe, because no adverse clinical or laboratory changes were observed (26). Serum levels increased up to normal levels, but the half-life of the infused MBL was variable. A few patients with recurrent debilitating infections clinically improved after MBL replacement therapy (27, 28).

We performed an open, uncontrolled safety and pharmacokinetic MBL-substitution study in 12 pediatric oncology patients with chemotherapy-induced neutropenia. In a cohort of 194 healthy Caucasian blood bank donors an optimal receiver-operator curve (ROC) cut-off value of 0.7 μg/ml MBL was calculated to divide individuals with a wild-type MBL2 genotype versus individuals with genetic variants (23). To achieve sufficient MBL-mediated complement activation and opsonophagocytosis, we aimed at a trough level MBL of 1.0 μg/ml. With twice weekly MBL infusions of 0.2 mg/kg (3-day interval) and 0.3 mg/kg (4-day interval), the median MBL trough level was 1.06 μg/ml. The half-life of the infused MBL was 36.4 hours (range 23.7-66.6 hours). Therefore, the pharmacokinetics were similar to those found in adults after adjustment for
bodyweight. None of the participating patients showed any adverse effect to the infused MBL and no anti-MBL antibodies were found four weeks after the last MBL infusion (29).

We investigated whether in vivo MBL substitution increased concomitant MBL-mediated in vitro complement activation, as measured by MBL-associated serine protease-2 (MASP-2), C3 and C4 activation, and opsonophagocytosis of zymosan as biological surrogate endpoints for the MBL serum reconstitution.

Material and Methods

Study design and protocol

A prospective, open, uncontrolled study was performed in 12 children (A-L) admitted to the pediatric oncology unit of the Emma Children’s Hospital, Amsterdam, the Netherlands for chemotherapy expected to induce neutropenia (<500 cells/µl). All children were ≤ 12 years of age and had a mutation in exon 1 of the MBL-2 gene. The study was conducted according to the declaration of Helsinki and Good Clinical Practice. The protocol was approved by the local ethics committee. All parents gave written informed consent in accordance with the Medical Research Involving Human Subjects Act (WMO). Twenty-four hours after the end of a chemotherapy course, patients received an MBL infusion (visit 1), which was repeated twice weekly (visit 2-6) until patients had recovered from chemotherapy-induced neutropenia (Figure 1). Patients were allowed to participate more than once. The dosage was 0.2 mg/kg MBL-SSI for a 3-day interval between infusions and 0.3 mg/kg MBL-SSI for a 4-day interval between infusions. We calculated the doses from a pharmacokinetic model based on the data from the phase-I study of Valdimarsson et al. (26). This was expected to increase the MBL serum concentration to ≥ 1.0 µg/ml, with a normalization of MBL-mediated opsonization. The study design and protocol, the MBL status and clinical characteristics of the 12 patients, as well as the pharmacokinetics and data on safety of the infused MBL-SSI are described in detail by Frakking et al. (29).

For this study, blood was sampled at several time points: before infusion, after 15 minutes, after 2, 4, 6, 16-24 hours, after 3-4 days (visit 2), before each next MBL infusion (visit 3-6) and 4 weeks after the last infusion. At all time points, MBL serum levels, MBL-mediated opsonization and clinical characteristics of the 12 patients, as well as the pharmacokinetics and data on safety of the infused MBL-SSI are described in detail by Frakking et al. (29).

MBL genotype and serum level

MBL levels and genotypes were determined at Sanquin Research, Amsterdam. Four ml of EDTA blood of all eligible patients was centrifuged for 10 minutes at 3400 rpm to separate plasma and buffycoat. DNA was isolated from white blood cells, by means of the QiAmp blood mini kit (Qiagen, Hilden, Germany). Twenty ng of DNA was used for the genotyping of each of the three
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exon-1 point mutations and for the three promoter polymorphisms by a Taqman assay with specific primers and minor-groove-binding probes as described by Brouwer et al.(23).

During the MBL-substitution study, blood was drawn, left to coagulate at room temperature and aliquots of serum were immediately stored at -80°C until tested. MBL serum levels were measured by ELISA technique as previously described (30). Briefly, mannan was coated to the solid phase and incubated with different dilutions of the sera. After washing, biotinylated monoclonal antibody MBL-1 (10 μg/ml, Sanquin, Amsterdam, the Netherlands) was used as detection antibody. After washing, plates were incubated with 1:10,000 diluted polymerized streptavidin-Horse Radish Peroxidase (HRP) for 30 minutes. The assay was developed with 100 μg/ml 3,3’,5,5’-tetramethylbenzidine (TMB) and the absorbance was measured at 450 nm.

**MBL production from human plasma**

The plasma-purified MBL used in this study was the first generation MBL-SSI, of which the purification has been described by Laursen et al. (24, 25). In short, the starting material for MBL purification was fraction II+III from the ethanol fractionation of plasma. Immunoglobulins were extracted from this product prior to MBL extraction, and an ultra-filtration performed to concentrate the extract. This was followed by affinity chromatography on Sepharose CL-4B and virus inactivation. Anion exchange chromatography on a Q Sepharose matrix and finally gel filtration on Superose 6 to exchange the buffer leading to the final MBL product. The purity of the product was about 70% and the major contaminants were IgM, serum amyloid P component, α2-macroglobulin and IgA. At 4°C the product is stable up to 3 years.

**Complement activation assays**

MBL-induced endogenous C3b and C4b deposition on mannan was assessed as described by Bultink et al. (31). Plates were coated overnight with 50 μg/ml of mannan. Dilutions of patient sera were incubated on the coated plates for 30 minutes at 37°C. After washing, the plates were incubated for 1 hour with 0.25 μg/ml biotinylated monoclonal antibody C3-19 (32) to measure C3b deposition or with 0.25 μg/ml biotinylated monoclonal antibody C4-10 (31) to measure C4b deposition. After washing, the plates were incubated with 1:10,000 diluted polymerized streptavidin-HRP for 30 minutes. The assay was developed with 100 μg/ml TMB and absorbance was measured at 450 nm. The results are presented in arbitrary units (AU), as compared to the mean C3 or C4 activation found in a poolserum of 3000 healthy control sera (MBL level 1.5 μg/ml), which was set at 100 AU. The assays are developed as very sensitive tools for MBL-dependent complement activation and are not sensitive for reduced C3 or C4 concentration in the sera (10% of the normal C4 / C3 concentration in serum is sufficient to detect normal C4 / C3 activation).

A C4b depositing activity assay to measure MASP-2 activation was performed with exogenous C4 at the department of Medical Microbiology and Immunology, University of Aarhus,
Denmark. In this assay, as described by Petersen et al. (33), the specific C4b-depositing capacity of the MBL pathway was determined by incubating serum diluted in buffer containing 2 mM CaCl₂ and 1 M NaCl in mannan-coated microtiter wells overnight at 4°C. After washing, wells were incubated with 0.1 μg of purified human C4 (34) for 1.5 hour, at 37°C to allow for the activation of C4 and the deposition of C4b onto the surfaces. Following a wash, deposited C4b was detected by adding 100 ng of biotinylated rabbit–antihuman C4c. C4b depositing activity was expressed as mU/ml, read from dilutions of standard plasma.

The activities of the classical, lectin and alternative pathways of complement were analyzed with the Wielisa kit (Wieslab, Lund, Sweden). This ELISA system is based on three different coatings (C1q, mannan and LPS, respectively). The read-out is determination of C5-C9 (35, 36).

**MASP-2 assays**

MASP-2 serum levels and MASP-2 binding capacity to MBL were determined at the department of Medical Microbiology and Immunology, University of Aarhus, Denmark. Due to limited patient material, only the samples before MBL infusion, 15 minutes after MBL infusion and at visit 2 were analyzed for MASP-2. Assays were performed as described by Møller-Kristensen et al. (37). Serum was incubated over night at 4°C, on anti-MASP-2 antibody (MAb 8B5, subclass IgG1)-coated microtiter wells to determine MASP-2 serum levels or on mannan-coated microtiter wells to determine MASP-2 binding capacity. MASP-2/Map19 binding was detected with 0.1 μg of biotinylated mouse-antihuman MASP-2/Map19 (6G12). MASP-2 serum levels are expressed in ng MASP-2/ml serum, MASP-2/Map19 binding capacity to MBL is expressed as mU/ml, both read from dilutions of standard plasma.

**Opsonophagocytosis assay**

The opsonophagocytosis assay has been described in detail by Brouwer et al. (23). Fresh isolates of neutrophils from healthy donors were purified from whole blood by centrifugation over a Percoll gradient as described by Roos and de Boer (38). After lysis of the erythrocytes, the neutrophils were washed in PBS and adjusted to 10⁷ cells/ml in HEPES medium (132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM HEPES), 2 mM CaCl₂, 5.5 mM glucose and 0.5% (w/v) human serum albumin (HEPES complete).

Zymosan, a mannan-rich insoluble cell-wall polysaccharide of *Saccharomyces cerevisiae* (lot no. 1389F, MP Biochemicals, Solon, OH, USA) was suspended in PBS at a concentration of 10 mg/ml and labeled for 30 minutes at 37°C with 10 μg/ml of FITC in the dark. After washing, the FITC-labeled zymosan was resuspended in PBS to 10 mg/ml. FITC-labeled zymosan (250 μg) was opsonized in 250 μl of 3% (v/v) human serum in HEPES complete, during 30 min of incubation at 37°C while shaking. Thereafter, the opsonized zymosan was washed twice and resuspended in 25 μl of HEPES complete. The classical pathway of complement was blocked with F(ab')₂, fragments of a monoclonal antibody against C1q (20 μg/ml C1q-85, Sanquin (39, 40)).
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Phagocytosis was initiated by mixing the opsonized FITC-labeled zymosan (25 μl) with neutrophils (0.5x10^6 cells) in HEPES complete to a final 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 stop buffer containing 0.5% (w/v) paraformaldehyde to stop phagocytosis.

Phagocytosis was determined by flow cytometry. 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 neutrophils). Non-phagocytized zymosan was excluded from analysis by gate R1. Results of the opsonophagocytosis assay are expressed as percentage of phagocytosis compared to a positive control (zymosan opsonized with MBL-sufficient serum), which was set at 100%. All samples were tested on three different days (n=3), with freshly isolated neutrophils.

\textit{Complement deposition on zymosan}

Oligomerization of the infused MBL and the binding of MBL and C3 to zymosan during opsonization with serum taken before and after MBL infusion, was investigated by Western blot analysis. Serum samples were prepared with 1% (v/v) serum in sample buffer (125 mM Tris, 20% (v/v) glycerol 5% (w/v) SDS and 0.02% (w/v) Coomassie blue) without 2-mercapto-ethanol or dithiotreitol. Zymosan was opsonized as described above, washed and resolved in HEPES medium without albumin and boiled at 95°C for 5 minutes in sample buffer. SDS-PAGE was performed on 5% (w/v) acrylamide gels with Precision Plus Protein\textsuperscript{TM} standard (Biorad) as molecular marker. Proteins from the SDS-PAGE gel were transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% (w/v) milk in 10 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20, pH 7.4 (TBST). After washing, the membranes were incubated in milk/TBST containing the primary monoclonal antibody (1:1000) anti-MBL-6 (Sanquin) or anti-iC3b (Quidel). After washing, the membranes were transferred to a 1:2500 secondary antibody dilution (goat-anti-mouse, GE Healthcare) in milk/TBST. Before detection with ECL Western blot reagent kit (Pierce), membranes were washed thoroughly with TBST and PBS.

\textit{Statistical analysis}

Changes in MBL levels and opsonophagocytosis of zymosan before and after MBL infusion were calculated with a non-parametric Wilcoxon signed-rank test for all 18 included observations. Calculation of only the first observation of the 10 included patients revealed similar data, but with less statistical power. The MBL level, opsonization of zymosan and MBL-mediated C3- and C4 activation were calculated for all visits (1-6). Data are expressed as mean ± standard error of the mean (SEM) in case of normally distributed data, unless otherwise mentioned, and as median (range) for not normally distributed data. Because of the limited number of patients, continuous variables were mainly presented by descriptive statistics.
Chapter 9

Results

Patient inclusions

The baseline patient characteristics are summarized in Table 1. Two patients did not meet all inclusion criteria and were excluded from statistical analysis (although inclusion of these two did not change the conclusions of the current dataset). Of the remaining 10 patients, six received MBL infusions repeatedly during one neutropenic episode and four received only a single MBL infusion, as schematically shown in Figure 1. Patient D was included four times, patient G and K three times and patient J two times. Each patient received a unique identification letter, and each inclusion a unique identification number. In total, 18 observations were included for analysis during this study (A01-L20).

MBL levels, complement activation and opsonophagocytosis: first 24 hours after infusion.

From 17 out of 18 included observations we followed the first 24 hours after the first MBL infusion. MBL levels, C3 and C4 activation were determined on solid-phase mannan to quantify the MBL binding and MBL/MASP-mediated complement activation. Zymosan opsonized with patient sera was phagocytized by control neutrophils to determine the opsonization capacity in the same blood samples, as biological read-out and in vitro surrogate marker for in vivo MBL reconstitution. The median MBL level, C3 and C4 activation and opsonophagocytosis of zymosan during the first 24 hours after MBL infusion are shown in Figure 2A.

MBL levels increased in all patients after the first infusion. Median MBL levels increased more than 10-fold from 0.38 μg/ml (range 0.03-1.69 μg/ml) before infusion (visit 1), to a peak level of 5.10 μg/ml (range 2.10-9.50 μg/ml) 15 minutes after infusion (Wilcoxon signed-rank,

<table>
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<th>ID</th>
<th>Age (years)</th>
<th>MBL2 genotype</th>
<th>MBL level (μg/ml)</th>
<th>Tumour</th>
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<td>2.1 HYPA/LYPB</td>
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<tr>
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<td>0.66</td>
<td>Common ALL</td>
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<td>M</td>
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<td>0.35</td>
<td>T cell ALL</td>
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<td>E</td>
<td>F</td>
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<tr>
<td>F</td>
<td>M</td>
<td>1.7 LXPA/LYPB</td>
<td>0.09</td>
<td>B-ALL</td>
<td></td>
</tr>
<tr>
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<td>M</td>
<td>0.5 HYPD/HYPD</td>
<td>0.08</td>
<td>Pro B-ALL</td>
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<tr>
<td>H</td>
<td>M</td>
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<td>0.09</td>
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<td>M</td>
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<td>0.54</td>
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</table>

Table 1. Patient characteristics

ID, identification letter; F, female; M, male; Age at the time of first MBL infusion; MBL level at the time of screening for inclusion; Tumor: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; GIST, gastro-intestinal stromal tumor; PNST, peripheral nerve sheath tumor; "T-cell lymphoblastic non-hodgkin lymphoma.
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Figure 1. MBL substitution regimen. Repetitive MBL infusions were given following a neutropenia-inducing chemotherapy course. The first MBL infusion was given 24 hours after the neutropenia-inducing chemotherapy, followed by MBL infusions every 3 or 4 days (each arrow represents one MBL infusion) until the patient was no longer neutropenic. Five patients received only a single infusion.

\textbf{Figure 2A}. MBL substitution regimen. Repetitive MBL infusions were given following a neutropenia-inducing chemotherapy course. The first MBL infusion was given 24 hours after the neutropenia-inducing chemotherapy, followed by MBL infusions every 3 or 4 days (each arrow represents one MBL infusion) until the patient was no longer neutropenic. Five patients received only a single infusion.

p<0.0001), declining to 2.50 \(\mu\)g/ml (range 1.23-4.00 \(\mu\)g/ml) in 24 hours (Fig. 2A, upper left panel).

The median MBL-mediated C3 and C4 activation and opsonophagocytosis of zymosan are also shown in Figure 2A. MBL-mediated C3 and C4 activation as well as the opsonophagocytosis increased after MBL infusion. MBL-dependent endogenous C4 activation showed an almost 4-fold increase directly after MBL infusion, whereas C3 activation was doubled. In contrast to the drastic increase in MBL, opsonophagocytosis of zymosan increased from 24\% (range 1-61\%) before to 75\% (range 8-130\%) directly after MBL infusion (Wilcoxon signed-rank, p<0.0003). Opsonization was also performed in the presence of F(ab)\_2 fragments of the inhibitory anti-C1q monoclonal antibody to block the classical pathway of complement activation (data not shown). This reduced the median opsonophagocytosis of zymosan before MBL infusion drastically to 6\%, which is in accordance with the fact that zymosan opsonization is largely MBL-dependent (23) and to some extent defined by endogenous anti-yeast/zymosan antibodies in the serum. After MBL infusion, there was only a 10-20\% reduction by the anti-C1q antibody compared to the opsonophagocytosis without these blocking antibodies (55\% opsonophagocytosis). The increase in opsonophagocytosis upon (repeated) MBL infusion showed an equal pattern with or without blocking anti-C1q antibodies and followed the pattern of the MBL level. MASP-2 serum levels remained equal before and after MBL infusions in all patients (supplemented Figure 1).

\textit{Complement activation and opsonophagocytosis: cumulative data after repeated infusion.}

At visit 2, just before the second infusion, the median MBL trough level was 0.90 \(\mu\)g/ml (0.26-1.84 \(\mu\)g/ml), significantly higher as compared with visit 1, before the first infusion of MBL (Wilcoxon signed-rank, p<0.0001). In the 12 patients who received repeated MBL infusions every 3 or 4 days (visit 2-6) during one neutropenic episode, we observed a cumulative effect of the repeated MBL infusions as MBL trough levels increased during the treatment (Figure 2B, upper
Although a similar increase was seen in the trough levels of C3 and C4 activation (Figure 2B, middle panels), the median trough levels in C3 and C4 activation remained below the levels achieved with a pool serum of 3000 healthy control sera (set at 100 AU; dotted line in Figure 2B).
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Opsonization was significantly higher at visit 2 as compared with opsonization at visit 1 (Wilcoxon signed-rank, *p*<0.025). Also the opsonization of zymosan (whether anti-C1q antibodies were added or not) showed a similar pattern as did the MBL level in those patients who received repeated MBL infusions. The median opsonophagocytosis increased from 24% before the first MBL infusion, to 45% before the MBL gift at visit 2 and to 83% at visit 5 (compared to control serum set at 100%, see dotted line in the lower panel of Figure 2B).

Because opsonophagocytosis was lagging behind during both the early and late stages of MBL reconstitution, we performed additional experiments to elucidate the achieved results. Figure 3 shows an *in vitro* titration of MBL-SSI to a serum of a healthy MBL-deficient control. This titration revealed that a higher level of MBL-SSI (3.3 μg/ml) was necessary to completely restore opsonophagocytosis of zymosan (to 100%) (figure 3A), compared to the serum MBL level (~ 1.3 μg/ml) necessary to achieve this 100% phagocytosis, in MBL-sufficient controls (Figure 3B).

*MBL oligomerization and binding capacity*

The MBL oligomerization and MBL binding to zymosan during the MBL substitution were determined with Western blot in the patient samples. As a representative for the study population, the longitudinal series of patient D04 are shown. The functional MBL levels, determined by binding to mannan-coated ELISA plates, peaked during the first 24 hours, followed by subsequent lower trough values for visits 2-5, as well as the normalized ‘four weeks after infusion’ sample (Figure 4A).

Not only the serum levels, but also the degree of MBL oligomerization in the sera (Figure 4B) showed a vast increase upon substitution during the peak phase and a dramatic shift from low-oligomeric MBL to high-oligomeric MBL, which remained consistently present during the whole substitution period. Prior to the MBL substitution, only very little MBL binding to zymosan was found, whereas the high-oligomeric MBL forms were detected after substitution (Figure 4B, lower Western blot). The ‘four weeks after infusion’ sample showed similar results as the samples taken before the first MBL infusion.

The response to repeated MBL infusions as assessed by trough levels of MBL, MBL-mediated C3 and C4 activation, and opsonophagocytosis of zymosan, of patient D and G with different inclusion periods, remained very similar within each patient, but varied considerably among these patients (Figure 5). Patient D showed in each of the courses of MBL substitution an increase in C3 activation and opsonophagocytosis, whereas patient G did not.

*MBL, complement activation and opsonophagocytosis before and after MBL substitution.*

Median levels of MBL-mediated endogenous C3 and C4 activation were above 100 AU during the first 24 hours after infusion, but these levels were not maintained in trough levels during repeated infusions. Although a significant increase in opsonophagocytosis was seen in all
patients 15 minutes after MBL infusion, compared to before infusion (Figure 6A), this increase was lower than expected based on the achieved MBL concentrations. MASP-2 binding to MBL on mannan-coated microtiter wells was increased upon MBL substitution and correlated ($r^2 = 0.8631$, $p<0.0001$) with the MBL levels achieved in the patients (Figure 6B). The MASP-2 activation, as measured by MBL-dependent C4b depositing activity with exogenous C4, did not correlate with MBL concentrations 15 minutes after MBL substitution (Figure 6C). Furthermore, compared to the correlation coefficient of MBL levels and MASP-2 activation in 100 control samples, the MASP-2 activation was suboptimal in the neutropenic patients both before and 15 minutes after MBL infusion, even though MASP-binding to MBL was not impaired in these samples.

To further investigate the suboptimal complement activation and the inter-individual variations detected for MBL-mediated C3 activation and opsonophagocytosis, the samples drawn at visit 1 (before the first MBL infusion) and 15 minutes after the MBL infusion from the patients were further analyzed. Western blots with eluates from opsonized zymosan visualized that MBL infusion in all ‘15 minutes after’ samples led to a reconstitution of functionally active high-oligomeric MBL molecules (trimer-pentamer) compared to the mainly low-oligomeric (dimer-trimer) MBL present in the eluates of zymosan incubated with serum drawn directly before the first MBL infusion (Figure 7A). The MBL levels, C3 deposition on zymosan analyzed with flow cytometry and opsonophagocytosis of these samples can be found in Figure 7B. The C3 deposition on zymosan showed variation before MBL infusion, but MBL infusion led to an increase in C3 deposition on zymosan in all cases. Upon MBL infusion, opsonophagocytosis of zymosan was increased, and opsonophagocytosis of zymosan in the samples 15 minutes after MBL infusion showed the same tendency as MBL-mediated C3 deposition in all but patient J14.
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Although CH50 and AP50 levels of the patients had been determined before participation to the MBL-substitution study, we reanalyzed all samples taken at visit 1, 15 minutes after MBL infusion and at visit 2 for the activation of the three complement pathways in a Wielisa and compared them with the positive control serum supplied with the Wielisa (Figure 7\( \text{C} \)). The mean activation of the classical pathway of complement in all samples was 90 ± 4% before and 84 ± 4% after MBL infusion. The mean lectin pathway activation was 33 ±7% in the samples drawn before MBL infusion. In the samples taken 15 minutes after MBL infusion an increase in the lectin pathway activation was seen but only up to 69 ± 6% of the positive control supplied with the kit, at a median MBL level of 5.1 μg/ml, and at visit 2 the lectin pathway activation was 27 ± 8%. This reduced lectin pathway activation can be well explained by the reduced MASP activation found in these patients. Although inter-individual variations were present, the alternative pathway was reduced (51 ±14% - 57 ± 6%) compared to the supplied control serum in almost all samples. G07, J14 and J16 showed no alternative pathway activation (3 ± 3% - 12 ± 2%) in the samples taken during the MBL substitution. However, in the samples taken from these patients 4 weeks after the study the alternative pathway activation was 54 ± 10% (Supplemented figure 2). These 3 observations with low alternative pathway activity also showed low C3 deposition on zymosan and low opsonophagocytosis by human neutrophils 15 minutes after MBL substitution. The opsonophagocytosis in patient J14 could be restored by mixing (1:1) this serum sample (15 min
after MBL infusion) with MBL-deficient serum from homozygous MBL-deficient controls having MBL levels under the detection limit of $< 0.05 \ \mu g/ml$. The exact nature of the temporary defects in this and the other patients has remained unidentified. Nonetheless, factor D deficiency was formally excluded, because purified factor D did not restore the alternative pathway activation in this patient, whereas the purified factor did do so when added to a control serum deficient for factor D (23).

**Discussion**

In this MBL-substitution study we demonstrate that the *in vitro* measured C3 complement activation and opsonophagocytosis of zymosan increased in all patients after *in vivo* administration of plasma-purified MBL. However, the recovery of opsonic function was suboptimal, both at the peak values of MBL during the first 24 hours and at the trough values before the next MBL infusion, although improvement was seen after repeated infusions.

Median MBL levels ranged from 5.1 $\mu g/ml$, 15 minutes after MBL infusion, to 2.5 $\mu g/ml$ 24 hours after MBL infusion. On average, MBL-mediated endogenous C3 activation seemed to be above the normal 100 AU. However, this assay was only sensitive for low MBL levels and not for
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... reduced C3 levels. C3 deposition on zymosan and the opsonophagocytosis of zymosan by normal control neutrophils did not reach the optimal 100% during this phase early after MBL infusion.

Several explanations for the suboptimal complement activation seen during the first 24 hours after MBL reconstitution were considered. *First*, we hypothesized that the 40% reduction in the complement-activating ability of MBL-SSI as a result of the purification process (24) could have limited opsonic activity. However, MBL levels are 5-10 times higher than the endogenous levels in MBL-sufficient individuals, and therefore this 40% reduction in MBL function cannot explain the low opsonization during the peak phase. This was further supported in the samples taken 15 minutes after MBL infusion, where *in vitro* addition of MBL-SSI did not lead to an increase in opsonophagocytosis of zymosan (data not shown). *Secondly*, we considered the possibility that MASP-2 binding and activation during the first 24 hours might be suboptimal or impaired. MASP-2, the most relevant MASP for complement-mediated opsonization, accounted for approximately 2.5% of the proteins present in the MBL-SSI product only (24, 25); thus, the MASP-2 necessary for lectin pathway activation is recruited from the circulation of the patients. MASP-2 binding to mannan-bound MBL highly correlated with MBL levels reached upon MBL substitution. However, the MASP-2 activation as analyzed by C4b depositing activity did not correlate with the achieved MBL levels and was much lower compared to MASP-2

![Figure 6](image_url)

Figure 6. The association between MBL levels and opsonophagocytosis of zymosan, MASP-2/Map19 binding capacity, and MASP-2 activation capacity determined as C4b depositing activity, in the patient sera before MBL infusion (visit 1) and 15 minutes after MBL infusion. Each symbol represents one sample. (A) Association between MBL level and opsonophagocytosis of zymosan. * Sera with low alternative pathway activation in the Wielisa. (B) Association between MBL level and MASP-2/Map19-binding to mannan-bound MBL. (C) Association between MBL level and MASP-2 activation (measured as C4b depositing activity) of the patient sera before (visit 1) and after MBL infusion (15 min), and 100 control sera (33, 43). Linear regression of the 100 control sera, r=0.98 (43).
activation in 100 control samples. Thus, although MASP-2 binding capacity was unimpaired, the proteolytic activity of MASP-2 may have been lost upon binding to C1 inhibitor or to α2-macroglobulin, some of the major impurities in the product. Malignancy-related or chemotherapy-

Figure 7. MBL and complement activation directly before and after the first MBL infusion. Samples from patient F, I, J, K and L drawn directly before (no mark) and 15 minutes after (*) the first MBL infusion were analyzed for MBL binding and complement activation. (A) Eluates from opsonized zymosan analyzed by SDS-page and immuno-blotting with anti-MBL antibodies. As a control, eluates from zymosan opsonized with PBS (-) and with plasma-purified MBL alone (MBL-SSI), were also run on the gel. (B) MBL level (upper panel), MBL-mediated C3 deposition on zymosan (middle panel) and opsonophagocytosis of zymosan (lower panel). (C) Complement pathway activation screened by Wielisa, read-out C5b-9 (n=16). Specific activation via the classical, lectin and alternative pathway of complement at visit 1, 15 minutes after MBL infusion and at visit 2 is given as means in % (+SD) of the positive control serum supplied with the Wielisa kit.
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induced reduction of MASP-2 proteolytic activity can also not be excluded. *Finally*, besides reduced proteolytic activity of MASP-2, we found a reduction of alternative pathway activation in the samples taken during the MBL substitution, when patients were neutropenic as a consequence of the chemotherapy. Although the reduction of alternative pathway activation varied among patients, alternative pathway activation seemed to be correlated with opsonophagocytosis of zymosan. We therefore conclude that the suboptimal opsonophagocytosis of zymosan was due to a temporary alternative pathway deficiency, possibly caused by reduced synthesis of complement proteins or increased consumption due to the severe neutropenia in these patients.

The doses of MBL had been predicted to yield trough levels of 1.0 µg/ml MBL, a level sufficient for opsonization in healthy control sera (29). Although this MBL trough level was reached, the levels of opsonophagocytosis were lower than expected. The loss of 40% of the complement-activating ability of MBL as described by Laursen (24) may provide (part of) the explanation for the suboptimal results of MBL-mediated C3 activation and opsonophagocytosis of zymosan at these sufficient MBL trough values. As was confirmed by dose-response curves of *in vitro* addition of MBL-SSI to MBL-deficient serum, a higher level of MBL is necessary to achieve the same opsonophagocytosis levels as compared to MBL in the circulation of normal MBL-sufficient donors. Taken into account this 40% reduction of complement-activation by plasma-purification of MBL, the calculated trough level of 1.0 µg/ml was apparently not sufficient for optimal complement activation and a higher dose may be considered to achieve sufficient activity of the infused MBL.

The inter-individual variations in opsonophagocytosis of zymosan after MBL substitution to similar trough levels are most likely caused by variations in other complement proteins rather than as a difference in effectiveness of the infused MBL-SSI. This can be concluded because the MASP-2 binding correlated well with the MBL concentration achieved after suppletion of MBL, and because of the very consistent complement activation between the different inclusions of the same individual (patients D, G, J and K). In the some of the patients included in this study, the alternative complement pathway activity seemed to be affected following chemotherapy, which can be rate-limiting in zymosan opsonization (23). Due to the limited amount of patient serum we have not been able to determine the limiting alternative pathway protein, but the defect was be restored by mixing of patient serum (1:1, v/v) with MBL-deficient control serum. Thus, the infused MBL-SSI from the patients’ serum could opsonize zymosan.

We studied whether MBL substitution increased MBL-mediated *in vitro* complement activation and opsonophagocytosis of zymosan as biological surrogate endpoint for the MBL serum reconstitution. Restoration of complement activation and opsonophagocytosis will be necessary to prevent infections in the neutropenic oncology patients. Because of the small number of patients, clinical efficacy could not be determined in this trial. However, MBL substitution has shown to be clinically beneficial in case reports (27, 41), and in pre-clinical studies with knock-out mice (42). A phase-II/III randomized controlled clinical trial is necessary to determine the clinical
effects of the MBL substitution in vivo.

In conclusion, this MBL-substitution study demonstrates that MBL substitution therapy with plasma-derived MBL not only restores MBL levels in vivo, but also increases MBL-mediated complement C3 activation and opsonophagocytosis of zymosan. However, the calculated trough level of 1.0 μg/ml MBL may not be sufficient to reach optimal opsonic function, due to the 40% loss of complement-activating ability during the purification of MBL. A higher trough level MBL may be necessary for in vivo efficacy. Despite the high MBL levels directly after MBL infusion, we found suboptimal complement activation and opsonophagocytosis of zymosan in this hemato-oncology cohort. This might be caused by defective MASP-2-mediated lectin pathway activation as well as the decrease in alternative pathway activity at the time of the MBL infusions in these patients. However, the decrease in alternative pathway activity and altered MASP-2 activity may not exist in other patient cohorts that may still potentially benefit from MBL suppletion. Because of the lack of phagocytes following the cycles of chemotherapy, the efficacy of MBL substitution therapy in this patient cohort may already be debated in the first place. According to our findings in a pediatric oncology cohort, other, preferably pediatric, patient cohorts should be targeted to demonstrate a therapeutic effect of MBL substitution in a phase-II/III randomized clinical trial. Premature neonates at the neonatal intensive care unit (NICU) may be a suitable target group. In these patients, MBL substitution may result in reduction of nosocomial infectious episodes, and in fewer admission days at the NICU.

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References

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Supplemented figures

**Supplemented Figure 1.** MASP-2 levels. Box plots represent the median (line), the 25-75% value (box) and the minimum and maximum values (error bars). The dotted line represents 100 AU of C4 activation. MASP-2 concentrations in the sera at visit 1, 15 minutes after MBL infusion and at through level before the next infusion (visit 2). Sample size varies from 15-17 samples.

**Supplemented Figure 2.** Complement pathway activation and opsonophagocytosis of zymosan of the patient sera with low alternative pathway activation (n=3). Specific activation via the classical, lectin and alternative pathway of complement at visit 1, 15 minutes after MBL infusion, at visit 2 and in the sample drawn 4 weeks after the last MBL infusion, screened by Wielisa, read-out C5b-9, as means in % of the positive control serum supplied with the Wielisa kit and opsonophagocytosis of zymosan expressed as % (± SEM) of the positive control, set at 100%.