Mannose-Binding Lectin (MBL) Substitution: Recovery of Opsonic Function In Vivo Lags behind MBL Serum Levels


J Immunol 2009; 183:3496-3504; Prepublished online 5 August 2009;
doi: 10.4049/jimmunol.0900445
http://www.jimmunol.org/content/183/5/3496

Supplementary Material: http://www.jimmunol.org/content/suppl/2009/08/05/jimmunol.0900445.DC1

References: This article cites 42 articles, 7 of which you can access for free at: http://www.jimmunol.org/content/183/5/3496.full#ref-list-1

Subscription: Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions: Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts: Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2009 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Mannose-Binding Lectin (MBL) Substitution: Recovery of Opsonic Function In Vivo Lags behind MBL Serum Levels

Nannette Brouwer,2* Florine N. J. Frakking,† Marianne D. van de Wetering,† Michel van Houdt,* Margreet Hart,† Ilona Kleine Budde,‡ Paul F. W. Strengers,‡ Inga Laursen,§ Gunnar Houen,§ Dirk Roos,*, Jens C. Jensenius,¶ Huib N. Caron,† Koert M. Dolman,*,† and Taco W. Kuijpers*†

Mannose-binding lectin (MBL)3 is a serum protein that belongs to the collectin family of proteins and has a structure similar to that of C1q. Both MBL and C1q are innate immune proteins that can initiate the complement cascade through associated proteases. C1q initiates the classical pathway of complement activation after binding to Abs, while MBL initiates the lectin pathway of complement activation after binding to repeating sugar structures on microorganisms (1, 2). The binding of MBL leads to opsonization of the microorganisms and a more efficient clearance by neutrophils. In 1989 the lack of opsonization of baker’s yeast (Saccharomyces cerevisiae) or its major capsular constituent zymosan was shown to be caused by MBL deficiency in the sera used (3). Presently, many associations between MBL deficiency and increased susceptibility to infection with various microorganisms have been published. MBL-deficient pediatric patients show a higher incidence of recurrent infections, mainly in the upper respiratory tract (4, 5). MBL deficiency is associated with a higher susceptibility to HIV infection (6, 7), reduced life expectancy in cystic fibrosis patients due to more frequent and more severe infections (8), and an increased risk of developing sepsis (9, 10). Currently, it is thought that only immunocompromised individuals also with MBL deficiency have an increased risk of infections, for example, neonates lacking adaptive immunity (11, 12), oncology patients receiving chemotherapy (13, 14), or individuals 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 (15–17).

Three structural point mutations in exon 1 of the MBL2 gene (D, B, and C variants, respectively) prevent formation of MBL oligomers and drastically reduce the MBL serum level. Additionally, three promoter polymorphisms (X/Y, H/L, and P/Q) exist. Due to linkage disequilibrium, only seven haplotypes are found, that is, HYPA, HYPD, HYQC, LXQA, LXPA, HYPD, and HYQC (18). In addition to the B, C, and D exon 1 variants, the LXPA haplotype is associated with decreased or deficient MBL levels (0.05–0.9 mg/ml) (19). The remaining individuals carrying the homozygous wild-type allele (A) show MBL levels >1.0 μg/ml up to 10 μg/ml. Thus, the MBL levels are largely genetically determined, but can differ up to 10-fold in individuals with identical genotypes for the six known variants (19). Previously, we found that in vitro addition of plasma-derived MBL (1.0–5.0 μg/ml) to MBL-deficient control sera completely

1 This work was supported by Landsteiner Stichting voor Bloedtransfusie Research (LSBR no. 0207).
2 Address correspondence and reprint requests to Dr. Nannette Brouwer, Medical Center Alkmaar, Laboratory KCHI, Julianna van Stolberglaan 13, 1814 HB Alkmaar, The Netherlands. E-mail address: n.brouwer@mma.nl
3 Abbreviations used in this paper: MBL, mannose-binding lectin; AU, arbitrary unit; MASP, MBL-associated serine protease; SSL, Statens Serum Institut.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

FIGURE 1. MBL substitution regimen. Repetitive MBL infusions were given following a neutropenia-inducing chemotherapy course. The first MBL infusion was given 24 h after the end of 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.

restored the opsonic function of these sera, as measured by the phagocytosis of opsonized zymosan by human neutrophils (20). Therefore, MBL-deficient patients with increased infection susceptibility might benefit from MBL substitution therapy. MBL was purified from plasma of Danish blood donors by Statens Serum Institut (SSI) (21, 22). In a phase I trial, MBL-SSI substitution appeared to be safe (23). Serum levels increased up to normal levels, but the half-life of the infused MBL showed variability. Some patients with recurrent debilitating infections clinically improved after MBL replacement therapy (24, 25).

We performed an open, uncontrolled safety and pharmacokinetic MBL-substitution study in 12 pediatric oncology patients with chemotherapy-induced neutropenia. The set-up of the study and the pharmacokinetics and the safety of the infused MBL have been described by Frakking et al. (26). In short, we aimed at an MBL trough level 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 through level was 1.06 μg/ml. The half-life of the infused MBL was 36.4 h (range, 23.7–66.6 h). None of the participating patients showed any adverse effects to the infused MBL, and no anti-MBL Abs were found 4 wk after the last MBL infusion (26).

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

Materials 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 at least one mutation in exon 1 of the MBL-2 gene. The study was conducted according to the Declaration of Helsinki and good clinical practice guidelines. 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 (Wet medisch-wetenschappelijk onderzoek). Twenty-four hours after the end of a chemotherapy course, patients received an MBL infusion (visit 1), which was repeated twice weekly (visits 2–6) until patients had recovered from chemotherapy-induced neutropenia (Fig. 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 MBL-SSI (23). 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. (26).

For this study, blood was sampled at defined time points: before infusion (visit 1); after 15 min, after 2, 4, 6, 16–24 h, after 3–4 days (visit 2); before each next MBL infusion (visits 3–6); and 4 wk after the last infusion. At all time points, MBL serum levels, MBL/MASP-mediated complement C3 and C4 activation, and opsonophagocytosis (in the absence or presence of a classical pathway blocking mAb against C1q) were analyzed.

MBL genotype and serum level

MBL levels and genotypes were determined at Sanquin Research (Amsterdam, The Netherlands). Four milliliters of EDTA blood of all eligible patients was centrifuged for 10 min at 3400 rpm to separate plasma and buffy coat. DNA was isolated from white blood cells, by means of the QIAamp blood mini kit (Qiagen). Twenty nanograms of DNA was used for the genotyping of each of the three 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. (27).

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 (27). Briefly, mannan was coated to the solid phase and incubated with different dilutions of the sera. After washing, biotinylated mAb MBL-1 (10 μg/ml, Sanquin) was used as detection Ab. After washing, plates were incubated with 1/10,000 diluted polymerized streptavidin-HRP (Sanquin) for 30 min. The assay was developed with 100 μg/ml 3,3′,5,5′-tetramethylbenzidine and the absorbance was measured at 450 nm.

MBL production from human plasma

The material used in this study was the first generation MBL-SSI Laursen (21) has described the purification of MBL from plasma. In short, the starting material for MBL purification was fraction II plus III from the ethanol fractionation of plasma. IgS were extracted from this product before MBL extraction, and an ultrafiltration was 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 final gel filtration on Superose 6 to exchange the buffer led to the final MBL product. The purity of the product was ≥70%, and the major contaminants were IgM, serum amyloid P component, α1-macroglobulin, and IgA. At 4°C the product is stable for up to 3 years. A 40% loss of specific complement-activating capacity (capacity per microgram of MBL) was seen during the purification process of MBL-SSI, which is explained by activation or dissociation of the MASP-2 protease from the MBL-MASP complex.

Complement activation assays

MBL/MASP-induced endogenous C3b and C4b deposition on mannan was assessed as described by Bultink et al. (28). Plates were coated overnight with 50 μg/ml mannan. Dilutions of patient sera were incubated on the coated plates for 30 min, at 37°C. After washing, the plates were incubated for 1 h with 0.25 μg/ml biotinylated mAb C3-19 (29) to measure C3b deposition or with 0.25 μg/ml biotinylated mAb C4-10 (28) to measure C4b deposition. After washing, the plates were developed as described above. The results are presented in arbitrary units (AU), as compared with the mean C3 or C4 activation found in a pool of 3000 healthy control sera (MBL level, 1.5 μg/ml), which was set at 100 AU. The assays are developed as very sensitive tests for MBL-dependent complement activation and are not sensitive for reduced native C3 or C4 concentration in the sera (10% of the normal C4/C3 concentration in serum is sufficient to detect normal C4/C3 activation by MBL-SSI/MASP-2).

A C4b-depositing assay to measure MASP-2 activity (i.e., MASP-2 activity test) 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. (30), the specific C4b-depositing capacity of the MBL pathway was determined by incubating serum samples...
diluted in buffer containing 2 mM CaCl₂ and 1 M NaCl in mannann-coated microtiter wells overnight at 4°C. After washing, the wells were incubated with 0.1 μg of purified human C4 (31) for 1.5 h 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 monoclonal anti-human-C4c. C4b-depositing activity was expressed as arbitrary mU/ml, read from dilutions of standard plasma.

The activities of the classical, lectin, and alternative pathways of complement were analyzed with the Wieslab kit (Wieslab). This ELISA system is based on three different coatings (IgM, mannan, and LPS, respectively). The read-out is the determination of deposited C5b-9. The results are given in percentage of the standard serum (set at 100%) supplied with the kit.

**MASP-2 assays**

MASP-2 serum levels and MASP-2 binding capacity of 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 min after MBL infusion, and at visit 2 were analyzed for MASP-2. Quantification of MASP-2 was performed by the assay described by Møller-Kristensen et al. (34), which measures total MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins.

**MASP-2 binding capacity of MBL**

MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins.

**Opsonophagocytosis assay**

The opsonophagocytosis assay has been described in detail by Brouwer et al. (20). 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 (35). After lysis of the erythrocytes, the neutrophils were analyzed for MASP-2. Quantification of MASP-2 was performed by the assay described by Møller-Kristensen et al. (34), which measures total MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins. Plasma samples diluted in a high salt, EDTA-containing buffer were incubated overnight at 4°C on anti-MASP-2 Ab (mAb 8B5)-coated microtiter wells and developed with biotinylated anti-MASP-2 Ab (6G12). To determine the amount of MASP-2 after dissociation from MBL and ficolins.

**Table I. Patient characteristics**

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>MBL2 Genotype</th>
<th>MBL Level (μg/ml)</th>
<th>Tumor</th>
<th>Reason for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>2.1</td>
<td>HYPA/LYPB</td>
<td>0.87</td>
<td>AML</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>M</td>
<td>8.1</td>
<td>LYQA/LYPD</td>
<td>0.66</td>
<td>Common ALL</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>12.5</td>
<td>LYQA/LXPA</td>
<td>0.35</td>
<td>T cell ALL</td>
<td>No exon 1 mutation</td>
</tr>
<tr>
<td>D</td>
<td>F</td>
<td>1.1</td>
<td>LYQA/LYPB</td>
<td>0.51</td>
<td>Neuroblastoma</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>9.7</td>
<td>LXPA/LYPB</td>
<td>0.48</td>
<td>Ewing sarcoma</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>M</td>
<td>1.7</td>
<td>LXPA/LYPB</td>
<td>0.09</td>
<td>B-ALL</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>M</td>
<td>0.5</td>
<td>HYPD/HYPD</td>
<td>0.08</td>
<td>Pro B-ALL</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>M</td>
<td>10.5</td>
<td>LXPA/LYPB</td>
<td>0.09</td>
<td>T cell lymphoma</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>M</td>
<td>15.4</td>
<td>LXPA/HYPD</td>
<td>0.47</td>
<td>GIST</td>
<td>Age</td>
</tr>
<tr>
<td>J</td>
<td>F</td>
<td>7.2</td>
<td>LXPA/HYPD</td>
<td>0.38</td>
<td>PNST</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>F</td>
<td>11.6</td>
<td>LXPA/LYPB</td>
<td>0.13</td>
<td>Osteosarcoma</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>M</td>
<td>9.6</td>
<td>LYPA/LYPB</td>
<td>0.54</td>
<td>Ewing sarcoma</td>
<td></td>
</tr>
</tbody>
</table>

* MBL indicates identification letter; F, female; M, male. Age is at the time of first MBL infusion. MBL level is at the time of screening for inclusion.

For tumors, AML indicates acute myeloid leukemia; ALL, acute lymphoblastic leukemia; GIST, gastrointestinal stromal tumor; PNST, peripheral nerve sheath tumor.

* T cell lymphoblastic non-Hodgkin lymphoma.

**Complement deposition on zymosan**

Oligomeric forms of the infused MBL and the binding of MBL and C3 to zymosan during opsonization with serum taken before and after MBL infusion were investigated by Western blot analysis. Samples were prepared with 1% (v/v) serum in nonreducing sample buffer (125 mM Tris, 20% (v/v) glycerol, 5% (v/v) SDS, and 0.02% (v/v) Coomassie blue). Zymosan was opsonized as described above, washed, and resolved in HEPES medium without albumin and boiled, at 95°C for 5 min, in sample buffer. SDS-PAGE was performed on 5% (w/v) acrylamide gels with Precision Plus Protein Standard (Bio-Rad) as molecular marker, and 10 μl of sample was loaded per lane. 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 Ab (1/10000) anti-MBL-6 (Sanquin) or anti-C3b (Quidel). After washing, the membranes were transferred to a 1/2500 secondary Ab dilution (HRP-conjugated 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.

**Statistical analysis**

Changes in MBL levels and opsonophagocytosis of zymosan before and after MBL infusion were calculated with a nonparametric 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. Differences in MASP-2 activation per microgram of MBL were calculated with a nonparametric Wilcoxon signed-rank test and the binding of MBL and C3 to zymosan during opsonization with serum taken before and after MBL infusion were investigated by Western blot analysis. Samples were prepared with 1% (v/v) serum in nonreducing sample buffer (125 mM Tris, 20% (v/v) glycerol, 5% (v/v) SDS, and 0.02% (v/v) Coomassie blue). Zymosan was opsonized as described above, washed, and resolved in HEPES medium without albumin and boiled, at 95°C for 5 min, in sample buffer. SDS-PAGE was performed on 5% (w/v) acrylamide gels with Precision Plus Protein Standard (Bio-Rad) as molecular marker, and 10 μl of sample was loaded per lane. 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 Ab (1/10000) anti-MBL-6 (Sanquin) or anti-C3b (Quidel). After washing, the membranes were transferred to a 1/2500 secondary Ab dilution (HRP-conjugated 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.

**Results**

**Patient inclusions**

The baseline patient characteristics are summarized in Table I. Two patients did not meet all inclusion criteria and were excluded.
MBL levels, complement activation, and opsonophagocytosis: the first 24 h after infusion

From 17 out of 18 included episodes, we followed throughout the first 24 h, after the first MBL infusion, the MBL levels and C3 and C4 activation on solid phase. Zymosan was opsonized with patient sera and 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, C4 and C3 activation, and opsonophagocytosis of zymosan during the first 24 h after MBL infusion are shown in Fig. 2A.

MBL levels increased in all patients after the first infusion. Median MBL levels increased >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 min after infusion (Wilcoxon signed-rank, \( p < 0.0001 \)), declining to 2.50 μg/ml (range, 1.23–4.00 μg/ml) in 24 h (Fig. 2A, upper left panel).

MBL/MASP-mediated C3 and C4 activation as well as opsonophagocytosis increased after MBL infusion (Fig. 2A). MBL/MASP-dependent endogenous C4 activation showed an ~4-fold increase directly after MBL infusion, whereas C3 activation was doubled. Because there was a wide range of MBL concentrations in the samples, the specific functional activity was also calculated afterwards per microgram of MBL to make a comparison possible between the samples drawn before infusion, 15 min thereafter, and at visit 2 after MBL infusion with each other and the samples of the control group (Table II). Unexpectedly, the complement C3 and C4 activating capacity per microgram of MBL before infusion was equal to that of the control serum, but declined after injection to ~40–50% of the control value.

Opsonophagocytosis of zymosan increased from 24% (range, 1–61%) before MBL infusion to 75% (range, 58–130%) 15 min after MBL infusion (Wilcoxon signed-rank, \( p < 0.0003 \)). The median percentage of opsonophagocytosis per microgram of MBL before MBL infusion was relatively low compared with the controls and reflected that of the specific complement activity by reducing further after MBL infusion (Table II). Opsonization was also performed in the presence of F(ab’\( ^{2} \)) fragments of the inhibitory anti-C1q mAb 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 (20). Thus, opsonophagocytosis in the absence of MBL is almost completely dependent on C1-mediated and Ig-dependent classical pathway activity determined by endogenous anti-yeast/zymosan Abs in the serum. This was also reflected in the samples taken 15 min after MBL infusion, where in the presence of anti-C1q Abs 55% opsonophagocytosis was achieved. Total MASP-2 serum levels remained equal before and after MBL infusions in all patients (supplemental Fig. 1).\(^4\)

\(^4\)The online version of this article contains supplemental material.

FIGURE 2. MBL level, C4 and C3 activation, and opsonophagocytosis of zymosan. Scatter plots of all included observations with the median (line) are included. The upper scatter plots show the MBL level, the central plots the MBL/MASP-mediated C4 and C3 activation, and the lower scatter plots the opsonophagocytosis of zymosan. Target MBL levels of 1.0 μg/ml, 100 AU C4 or C3 activation, and 100% phagocytosis are depicted with dotted lines. A, Samples taken during the first 24 h after the first MBL infusion. The first sample was drawn before MBL infusion (pre) and the other samples at 15 min and 2, 4, 6, and 24 h after the MBL infusion. Sample size varies from 15 to 17 samples. B, Samples taken before every MBL infusion to determine the trough levels. Visit 1 is the sample drawn before the first MBL infusion (pre); the other visits took place every 3–4 days. Sample size varies due to differences in neutropenic periods among patients.

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 μg/ml (0.26–1.84 μg/ml), significantly higher as compared with visit 1, before the first infusion of MBL (Wilcoxon signed-rank, \( p < 0.0001 \)). In the 12 included observations of patients who received repeated MBL infusions every 3 or 4 days (visits 2–6) during one single neutropic episode, we observed a cumulative effect of the repeated MBL infusions during such a...
single neutropenic episode, as MBL trough levels increased during the treatment (Fig. 2B, upper panel). Although a similar increase was seen in the trough levels of C4 and C3 activation (Fig. 2B, middle panels), the median trough levels of C3 and C4 activation remained below the levels achieved with a serum pool of 3000 healthy control sera (set at 100 AU; dotted line in Fig. 2B). However, the median specific C4- and C3-activating capacities at visit 2 were equal to the activation achieved with the control serum pool: 61 vs 67 AU/µg MBL for C4, and 78 vs 67 AU/µg MBL for C3 (Table II).

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 Abs were added or not) showed an increasing pattern, comparable to the MBL level in those patients who received repeated MBL infusions. The median opsonophagocytosis before the MBL infusions at each visit increased from 24% at visit 1, to 45% at visit 2, and to 83% at visit 5 (compared with control serum set at 100%; see dotted line in the lower panel of Fig. 2B). However, when calculating the median opsonophagocytosis per microgram of MBL, the opsonophagocytosis capacity was 63%/µg MBL at visit 1, 50%/µg MBL at visit 2, and 52%/µg MBL at visit 5 vs 75% for the control (Table II).

Fig. 3 shows an in vitro titration of MBL-SSI added to a serum of a healthy MBL-deficient control individual. This titration revealed that a 2.5-fold higher level of MBL-SSI (3.3 µg/ml) was necessary to completely restore opsonophagocytosis of zymosan (to 100%) (Fig. 3A), compared with the serum MBL level (~1.3 µg/ml) necessary to achieve this 100% phagocytosis in MBL-sufficient controls (Fig. 3B). Because the 40% loss of specific complement-activating capacity (capacity per microgram of MBL) during the purification process of MBL-SSI (21) could not fully explain the results the first 24 h after infusion of MBL, we performed additional experiments aimed at elucidating the observations.

### Table II. Specific activation of MASP, C4, C3, and opsonization per microgram of MBL

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activation per Microgram of MBL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MASP-2 (mU)</td>
</tr>
<tr>
<td>Pre</td>
<td>268</td>
</tr>
<tr>
<td>15 min</td>
<td>99</td>
</tr>
<tr>
<td>Visit 2</td>
<td>Not determined</td>
</tr>
<tr>
<td>Controls</td>
<td>586</td>
</tr>
</tbody>
</table>

*Values are the median of each group. For controls, MASP-2 includes 100 healthy volunteer donors; C4/C3, pool serum of 3000 sera; opsonization, MBL-sufficient donor sera used in all opsonization assays.

MBL oligomeric forms and binding capacity

The distribution of MBL oligomeric forms and MBL binding to zymosan during the MBL substitution were determined with Western blot of the patient samples. With the increase in MBL serum levels upon substitution, the presence of MBL oligomers in the sera showed a vast increase and a dramatic shift from low-oligomeric MBL to high-oligomeric MBL, which remained consistently present during the whole substitution period. This was also reflected in the amount of MBL bound to zymosan. Supplementary Fig. 2 shows the longitudinal series of patient D04, as a representative for the study population.

The response to repeated MBL infusions as assessed by trough levels of MBL, MBL/MASP-mediated C3 and C4 activation, and opsonophagocytosis of zymosan, of patients D and G with different inclusion periods, remained very similar within each patient, but showed considerable interpatient variation (Fig. 4). Patient D showed in each of the courses of MBL substitution an increase in C3 activation and concomitant opsonophagocytosis, whereas patient G did not. Apparently, interindividual variations in patients’ complement activation capacity were playing a role in the results achieved by MBL infusion.

The role of the alternative pathway amplification loop

To further investigate the suboptimal complement activation and the interindividual variations detected for MBL/MASP-mediated complement activation and opsonophagocytosis, the samples drawn before MBL infusion (visit 1) and 15 min after the MBL infusion were further analyzed. Western blots loaded with eluates from opsonized zymosan visualized that MBL infusion in all “15 min after” samples led to a reconstitution of functionally active high-oligomeric MBL molecules (trimer-pentamer) compared with the mainly low-oligomeric (dimer-trimer) MBL present in the eluates of zymosan incubated with serum drawn directly before the first MBL infusion (a representative group of samples is shown in Fig. 5A). The MBL levels, C3 deposition on zymosan analyzed by flow cytometry, and opsonophagocytosis of these samples can be found in Fig. 5B. 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. A comparable increase was obtained for opsonophagocytosis of zymosan upon MBL infusion in all but one patient (J14).

Although CH50 and AP50 levels of the patients had been determined before participation in the MBL-substitution study, we reanalyzed the activation of the three complement pathways in all samples taken at visit 1, 15 min after MBL infusion, and at visit 2 with a Wielisa (expressed as percentage of the positive control serum, Fig. 5C). The mean activation of the classical pathway of

---

**FIGURE 3.** Phagocytosis of zymosan opsonized with increasing concentrations of MBL. A. Phagocytosis by neutrophils of zymosan opsonized with an MBL-deficient serum, supplemented by a titration series of MBL-SSI in vitro. B. Phagocytosis by neutrophils of zymosan opsonized with a series of MBL-sufficient sera, with the MBL concentration of each serum at the x-axis. Each bar represents the mean ± SEM of three independent measurements.
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 min after MBL infusion, an increase in the lectin pathway activation was seen, but only to a mean of 69 ± 6% of the positive control, at a median MBL level of 5.1 μg/ml. At visit 2 the mean lectin pathway activation was 27 ± 8%. Although interindividual variations were present, the alternative pathway was reduced (51 ± 14% to 57 ± 6%) compared with the supplied control serum in almost all samples (p < 0.05). G07, J14, and J16 showed no alternative pathway activation (3 ± 3% to 12 ± 2%) in the samples taken during the MBL substitution. However, in the samples taken from these patients 4 wk after the study the alternative pathway activation was 54 ± 10% (supplemental Fig. 3). These three observations of low alternative pathway activity also showed low C3 deposition on zymosan and low opsonophagocytosis by human neutrophils 15 min after MBL substitution (Fig. 6A, asterisks). The opsonophagocytosis in patient J14 could be restored by mixing (1/1) this serum sample (15 min after MBL infusion) with MBL-deficient pool serum from MBL-deficient controls having MBL levels under the detection limit of <0.05 μg/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 sample, whereas it did so when added to a control serum deficient for factor D (20).

MASP-2 activity in oncology patients

Although a significant increase in lectin pathway activation (Wielisa, Fig. 5C) and opsonophagocytosis of zymosan (all measured three times at 3 different days) from patient D (upper panels, D04, D08, and D09) and patient G (lower panels, G07, G13, and G18) who received MBL infusions during three different neutropenic episodes. }

There was a significant increase in complement activation and opsonophagocytosis in all patients after in vivo administration of plasma-derived MBL. However, the recovery of opsonic function was suboptimal, especially at the peak values of MBL during the first 24 h and to a lesser extent at the trough values before the next

Discussion

In this MBL-substitution study we demonstrate that the in vitro measured complement activation and opsonophagocytosis of zymosan increased in all patients after in vivo administration of plasma-derived MBL. However, the recovery of opsonic function was suboptimal, especially at the peak values of MBL during the first 24 h and to a lesser extent at the trough values before the next
MBL infusion, although improvement was seen after repeated infusions. Because of the small number of patients, the different types of malignancies (as given in Table I), and consequently the different chemotherapies these patients received during this trial, it was impossible to determine the clinical efficacy of MBL substitution.

The doses of MBL had been predicted to yield trough levels of $1.0 \mu g/ml$ MBL, a level considered sufficient for opsonization in healthy control sera (26). Although the MBL trough level was reached, the levels of opsonophagocytosis were lower than expected. Even though a loss of 40% of the complement-activating capacity during the plasma purification of MBL-SSI has been described (21), this can only provide part of the explanation for the suboptimal results of MBL/MASP-mediated C3 and C4 activation and opsonophagocytosis of zymosan at the increased MBL peak values.

As indeed indicated by dose-response curves of in vitro addition of MBL-SSI to MBL-deficient serum of healthy controls, a higher level of MBL-SSI is necessary to achieve the same opsonophagocytosis levels as compared with MBL in the circulation of normal MBL-sufficient donors. Therefore, the calculated trough level of $1.0 \mu g/ml$ was apparently not sufficient for optimal complement activation and a higher dose may be considered to achieve sufficient activity from infused MBL in this patient group. However, even at peak levels of MBL with a median level of $5.1 \mu g/ml$ following substitution, the ratios of C3 and C4 activation and opsonophagocytosis of zymosan per microgram of MBL (at 15 min after infusion) were only half of those of the complement activation per microgram of MBL in control sera. Furthermore, in vitro addition of MBL-SSI in these samples taken 15 min after MBL infusion did not lead to any additional increase in opsonophagocytosis of zymosan (data not shown), which indicates limitations in or exhaustion of the endogenous native complement supply in these patients. The interindividual variation in opsonophagocytosis of zymosan after MBL substitution to similar trough levels also supports the idea that the variation is caused by insufficiency of other complement proteins rather than a difference in effectiveness of the infused MBL-SSI. This is further supported by the correlation between MASP-2 binding to MBL and the MBL concentration achieved after suppletion, as well as by the very consistent complement activation ratio between the different periods of MBL suppletion within the same individuals (patients D, G, J, and K).

MASP-2 activation was suboptimal in our oncology patients. MASP-2 is the most relevant MASP for complement activation and accounts for $\approx 2.5\%$ of the proteins present in the MBL-SSI product, that is, $\approx 17\%$ of the MBL oligomers are associated with MASP-2 dimers (21, 22). Thus, MASP-2 necessary for lectin pathway activation may be recruited from the circulation of the patients. Although MASP-2 concentrations in the blood were normal, the enzymatic activity was much lower in oncology patients (irrespective of treatment and/or MBL substitution) than the MASP-2 activity determined in 100 healthy controls (30, 38). The explanation may be related to the presence of protein inhibitors such as C1 esterase inhibitor, $\alpha_2$-macroglobulin, or as yet unidentified modulators of its activity (39). Also, the reduction of alternative pathway activation in samples from several of the oncology patients,

**FIGURE 5.** MBL and complement activation directly before and after the first MBL infusion. Samples from patients F, I, J, K, and L drawn before (no mark) and 15 min after (*) the first MBL infusion were analyzed for MBL binding and complement activation. A. Eluates from opsonized zymosan analyzed by SDS-PAGE and immunoblotting with anti-MBL Abs. Eluates from zymosan opsonized with PBS (−) and with plasma-derived MBL alone (MBL-SSI) were run on the gel as controls. B, MBL level (upper panel), MBL-mediated C3 deposition on zymosan (middle panel), and opsonophagocytosis of zymosan (lower panel) before and after MBL infusion. C, Complement pathway activation screened by Wielisa (read-out C5b-9, n = 16). Specific activation via the classical, lectin, and alternative pathways of complement at visit 1, 15 min after MBL infusion, and at visit 2 is given as means in percentage (±SD) of the positive control serum supplied with the Wielisa kit.
increased consumption due to the chemotherapy-induced tissue damage and bone marrow suppression in these patients. In summary, malignancy-related or chemotherapy-induced reduction of both the MASp-2 and alternative pathway activity was unexpected and needs to be addressed in future studies.

In conclusion, our MBL substitution study demonstrates that plasma-derived MBL restores serum levels in vivo. The infused MBL increases MBL/MASP-mediated complement activation and opsonophagocytosis of zymosan in vitro, albeit to a suboptimal extent. The calculated trough level of 1.0 μg/mL MBL (26, 38) is not sufficient to reach optimal serum opsonic function, which cannot be explained only by the 40% loss of C4-activating ability during purification of MBL from plasma (21). MBL substitution has shown to be clinically beneficial in patients treated on compassionate grounds as presented in case reports (40, 41) and in preclinical studies with knockout mice (42), without chemotherapy treatment. The efficacy of MBL substitution therapy in oncology patients may be disputed, because the lack of phagocytes following chemotherapy prohibits efficient removal of (opsonized) microorganisms. Additionally, in our cohort some patients seem to have additional complement defects in lectin and alternative pathways that may have accounted for an increased infection risk due to inefficient opsonophagocytosis. A phase II/III randomized placebo-controlled clinical trial is necessary to determine the clinical efficacy of the MBL substitution in vivo.

Acknowledgments

We thank Alwin D. Huitema for the calculations of the pharmacokinetics and Henriët Nienhuis for helping with statistical analyses. Ed Nieuwenhuys and the diagnostic Department of Immunochefistry (Sanquin) are acknowledged for their help with determination of MBL levels. Steffen Thiel and Annette Gudmann Hansen are greatly acknowledged for their helpful suggestions and analysis of MASp-2 serum levels, MASp-2 binding, and MASp-2 activation. We further thank Nelia Langeveld for practical help during the MBL infusions, and the oncologists of the Emma Children’s Hospital Academic Medical Centre for their help in recruiting patients. MBL-SSI was a gift from Statens Serum Institute, Copenhagen, Denmark.

Disclosures

G. Houen and I. Laursen work at the Statens Serum Institut (Denmark), where the plasma-derived MBL was produced. The study was performed and written by researchers of Sanquin Research and the Emma Children’s Hospital (Academic Medical Centre), Amsterdam, The Netherlands. These researchers do not have a conflict of interest to declare.

References


