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
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, HYP, LYP, LYQA, LXP, HYPD, LYPB, and LYQC (18). In addition to the B, C, and D exon 1 variants, the LXP haplotype is associated with decreased or deficient MBL levels (<0.05 μg/ml) (18). 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...
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.

The study design is as follows:

**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 anduffy 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, mannann 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. Igswere extracted from this product before MBL extraction, and an ultraffltration 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 finally 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 tools 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 mannan-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 Wielisa kit (Wieslab). This ELISA system is based on three different coatings (IgM, mannan, and LPS, respectively). The read-out is the determination of deposited C4b9 (32, 33). 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 (mAb 6G12). To determine the amount of MASP-2 bound in MBL/MASP-2 complexes, plasma was 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 (6G12). To determine the amount of MASP-2 and Map19 in the wells. After washing, the wells were developed with biotinylated anti-MASP-2 Ab dilution (HRP-conjugated goat anti-mouse; GE Healthcare) in (Quidel). After washing, the membranes were transferred to a 1/2500 sec-

**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 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 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 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 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 purified. The baseline patient characteristics are summarized in Table I. Two patients did not meet all inclusion criteria and were excluded from analysis by gate R1. Results of the opsonophagocytosis assay were expressed as percentage of phagocytosis compared with a positive control (zymosan opsonized with MBL-sufficient serum with 1.3 μg MBL/ml), which was set at 100%. Adhesion of particles to the neutrophils is minimal if any and does not play any role, as shown before (20). All samples were tested on three different days (n = 3) with freshly isolated neutrophils.

**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% (v/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% (v/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 (Fierce), 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 multiple regression and test. The MBL level, opsonization of zymosan and MBL/MASP-2-mediated C3 and C4 activation were calculated for all visits (1–6). Data are expressed as means ± 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.

**Results**

**Patient inclusions**

The baseline patient characteristics are summarized in Table I. Two patients did not meet all inclusion criteria and were excluded from analysis by gate R1. Results of the opsonophagocytosis assay were expressed as percentage of phagocytosis compared with a positive control (zymosan opsonized with MBL-sufficient serum with 1.3 μg MBL/ml), which was set at 100%. Adhesion of particles to the neutrophils is minimal if any and does not play any role, as shown before (20). All samples were tested on three different days (n = 3) with freshly isolated neutrophils.
from statistical analysis (although inclusion of these two did not change the conclusions of the current data set). Of the remaining 10 patients, 6 received MBL infusions repeatedly during one or more neutropenic episodes, while 4 received only a single MBL infusion, as schematically shown in Fig. 1. Patient D was included through four episodes, patients G and K for three episodes, patient J for two episodes, and patients A, B, E, F, and L each for one episode. 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: 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, 8–130%) 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 \( \frac{\text{Fab}^\prime}{\text{Fc}} \) 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

**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 neutropenic episode, we observed a cumulative effect of the repeated MBL infusions during such a
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. Supplemental 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

---

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>MASP-2 (mU)</th>
<th>C4 (AU)</th>
<th>C3 (AU)</th>
<th>Opyonization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>268</td>
<td>66</td>
<td>145</td>
<td>63</td>
</tr>
<tr>
<td>15 min</td>
<td>99</td>
<td>28</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>Visit 2</td>
<td>Not determined</td>
<td>61</td>
<td>78</td>
<td>50</td>
</tr>
<tr>
<td>Controls</td>
<td>586</td>
<td>67</td>
<td>67</td>
<td>75</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.
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 do 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.

**FIGURE 4.** Repeated inclusion of two patients during different neutropenic episodes. From left to right: Average MBL level, MBL/MASP-mediated C3 activation, 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.
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 μg/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 μg/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 μg/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 supplementation, as well as by the very consistent complement activation ratio between the different periods of MBL supplementation 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 ~2.5% of the proteins present in the MBL-SSI product, that is, ~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, α2-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,
when these patients were neutropenic as a consequence of the chemotherapy, may have had an effect. Low alternative pathway activity may be rate-limiting in zymosan opsonization (20). In this study, low alternative pathway activity seemed indeed to correlate with the patient’s reduced opsonophagocytosis of zymosan. Four weeks after MBL infusion (when patients were no longer neutropenic), the alternative pathway activity was increased but still suboptimal. The temporary alternative pathway deficiency may be caused by the reduced synthesis of alternative pathway complement proteins from liver toxicity 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 Immunochemistry (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

3504 RECOVERY OF OPSONIC FUNCTION AFTER MBL SUBSTITUTION


Supplementary Figure 1. MASP-2 serum levels.

Scatter plots with the median (line) of MASP-2 concentrations in the sera at visit 1, 15 min after MBL infusion and at trough level before the next infusion (visit 2). Sample size varies from 15-17 samples.
Supplementary Figure 2. MBL serum levels, oligomeric forms and MBL and C3 deposition on zymosan.

Representative MBL levels of patient D during the first 24 hours after MBL substitution, before every MBL infusion (visit 1-5) and in the sample taken 4 weeks after the last MBL infusion. Figure S2A shows the MBL level as determined with ELISA. The upper Western blot of Figure S2B shows the oligomeric forms of the infused MBL, while the lower Western blot shows the MBL binding capacity to zymosan. Figure S2C shows the C3 deposition (AU) on zymosan (with MBL-sufficient control sera set at 100 AU).
Supplementary Figure 3. Complement pathway activation and opsonophagocytosis of zymosan of the patient sera with low alternative pathway activation.

Specific activation via the classical, lectin and alternative pathway of complement at visit 1, at 15 min after MBL infusion, at visit 2, and in the sample drawn 4 weeks after the last MBL infusion. This was screened by Wielisa, read-out C5b-9, as means in % of the positive control serum supplied with the Wielisa kit, and by opsonophagocytosis of zymosan expressed as % of the positive control, set at 100%.
Supplementary Figure 4: Association of MBL serum levels and MASP-2 activation in 34 pediatric oncology patients compared to healthy control sera (30, 39).

Patients were screened for MBL genotype and serum levels but not included in this substitution study (13, 26). Each symbol represents one sample. Linear regressions of the patient sera; $r^2=0.5603$, and of the control sera; $r^2=0.9142$ (slope significantly different, p<0.0001).
Graph showing MASP-2 levels (ng/ml) at different times after infusion:

- Pre
- 15 min.
- Visit 2

The graph displays variability in MASP-2 levels across these time points.