Mannose-Binding Lectin Is a Critical Factor in Systemic Complement Activation during Meningococcal Septic Shock

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Background. Systemic activation of complement during meningococcal disease is associated with severe disease and poor outcome. The exact mechanism of activation of complement is unknown but is important for future therapies aimed at modulating the complement system in this disease.

Methods. We studied complement activation in a group of 22 patients, including 18 with meningococcal septic shock and 4 with meningococcal disease without shock. Two of the patients with shock were MBL deficient: 1 patient was homozygous and 1 patient was compound heterozygous for exon 1 mutations in the gene for MBL.

Results. The MBL-deficient patients had relatively low disease severity and mild disseminated intravascular coagulation (DIC). At admission to the pediatric intensive care unit, the MBL-deficient patients had much lower circulating values of C3bc (indicating common pathway activation) and terminal complement complex (indicating terminal pathway activation) than did MBL-sufficient patients who presented with meningococcal septic shock. Levels of C4bc (indicating classical or lectin pathway activation) and C3bBbP (indicating alternative pathway activation) were also decreased in the MBL-deficient patients. Systemic activation of complement excellently correlated with disease severity and parameters of DIC. Testing of convalescent blood samples from 1 of the MBL-deficient patients in a model of meningococcal sepsis showed that a lack of lectin pathway activation leads to a reduced activation of complement.

Conclusions. This indicates that MBL is critical for the systemic activation of complement seen during meningococcal septic shock.

Meningococcal septic shock (MSS) is a lethal disease that can kill previously healthy children within a 24-h period [1]. The complement system plays a complex dual role in the pathogenesis of MSS. It is essential for the early defence against Neisseria meningitidis [2, 3]. On the other hand, extensive systemic complement activation is associated with severe disease and poor outcome [4, 5].

Complement is activated on the surfaces of meningococci or meningococcal outer membrane blebs by 1 or more of the 3 initial complement-activating pathways: the classical pathway, the lectin pathway, and the alternative pathway. Antibody, C-reactive protein, and complement factor (C) C1q mediate classical pathway activation. Mannose-binding lectin (MBL) is the central molecule in the lectin pathway of complement activation. MBL deficiency (ie, undetectable levels of MBL in plasma) occurs when a person is homozygous (or compound heterozygous) for any of the 3 structural mutations in exon 1 of the MBL gene; heterozygosity for these mutations leads to reduced plasma MBL concentrations. MBL deficiency is relatively frequent and is linked to increased susceptibility to certain infectious diseases [9, 10], although its

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We studied complement activation, cytokine production, and disease severity in a group of 22 patients with invasive meningococcal disease. Two of these patients were deficient for MBL, which is the central molecule in the lectin pathway of complement activation.

**PATIENTS AND METHODS**

From 2001 through 2006, 25 children with meningococcal disease were admitted to the pediatric intensive care unit (PICU) of the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands). The PICU of the Radboud University Nijmegen Medical Centre receives patients either as direct admissions from the emergency department or after referral by other hospitals. Material from 22 of these patients was available for analysis. Informed consent was given for each patient by the parents, and the study was approved by the local ethics committee and performed according to local guidelines. Diagnosis of meningococcal disease was made on the basis of positive culture results or positive results of gram staining of blood, cerebrospinal fluid, or skin biopsy samples (82% of cases) or on the basis of typical presentation (18%). All isolated meningococci were serogroup B (the study was started after the national vaccination campaign against group C meningococci). Eighteen patients presented with septic shock (shock group), and 4 patients presented without shock (1 had meningococcal bacteremia, and 3 had meningococcal meningitis). Shock was defined according to the Consensus Conference Definitions for Sepsis and Organ Dys-function in Pediatrics [15]. Disseminated intravascular coagulation (DIC) scores were calculated according to the scoring system of the International Society for Thrombosis and Haemostasis [16]. All of the patients received prompt antibiotic therapy (ceftriaxone administered intravenously) and dexamethasone during the first 3 days of hospitalization. Fresh frozen plasma (FFP) was given to 14 patients with septic shock and signs of DIC. One of the patients received plasma exchange, which is the central molecule in the lectin pathway of complement activation.
Figure 1. Complement activation products at admission to pediatric intensive care unit for patients with meningococcal disease. C3bc and terminal complement complex (TCC) reflect common pathway and terminal pathway activation, respectively, C4bc reflects classical or lectin pathway activation, and C3bBbP reflects alternative pathway activation. Lines indicate medians values. Differences between groups were tested for statistical significance using the Mann-Whitney U test. A/A, mannose-binding lectin wild type; A/O, heterozygous exon 1 mutation; O/O, homozygous exon 1 mutation.

the 2 MBL-deficient patients (patient 1) received no FFP, whereas the other MBL-deficient patient (patient 2) received FFP before admission to our PICU. Among the patients without MBL deficiency, 3 received recombinant tissue plasminogen activator as adjunctive treatment. Pediatric risk of mortality (PRISM) and DIC scores were calculated retrospectively by one of the researchers (T.S.) [17, 18].

For determination of complement activation, ethylenediaminetetraacetic acid (EDTA)–anticoagulated blood was obtained at admission to the PICU and at 2, 8, 24, and 48 h after admission. Heparin-anticoagulated blood was obtained for determination of cytokine concentrations. Samples were immediately put on ice, centrifuged for 15 min at 2800 g, and frozen at −80°C until analysis in batch.

One of the MBL-deficient patients (patient 1) and an age- and disease-matched control subject who had had meningococcal meningitis were invited, 1 year after experiencing their episodes of meningococcal disease, for an additional in vitro whole blood stimulation experiment [8]. In brief, lepirudin anticoagulated whole blood was incubated for 1 h with 10⁶ bacteria/mL of heat-killed serogroup B N. meningitidis (strain H44/76 [19]) at 37°C, whereafter 25 mM EDTA was added, put on ice, centrifuged, and frozen at −80°C until assay for the complement intermediates. Patient 2 and his parents did not give permission to draw blood for additional experiments after convalescence because of extreme fear regarding venipuncture in this patient.

Complement activation products were measured by enzymelinked immunosorbent assay (ELISA), as described elsewhere in detail. C1r-C1inh complexes (classical pathway) [20], C4bc (classical and lectin pathway) [21], and C3bBbP (alternative pathway) [22] were used as markers of initial pathway activation; C3bc [23] and TCCs [24] indicate final common pathway or total complement activation. Cytokines (tumor necrosis factor [TNF]–α, interleukin [IL]–1β, and IL-10) were determined using commercially available Bio-Plex protein array system (Bio-Rad Laboratories) [25]. MBL plasma concentration was measured by ELISA (AntibodyShop). MBL genotype was determined using restriction fragment–length polymorphism (RFLP) analysis for the structural mutations in exon 1 of the MBL gene [26, 27].

RESULTS

Eighteen patients presented with shock. Disease characteristics are presented in Table 1. One patient (patient 1) was identified as homozygous for a point mutation in codon 54 of exon 1 of the MBL gene and had undetectable plasma concentrations of MBL at PICU admission. A second patient (patient 2) was compound heterozygous for mutations in codon 52 and 54 of exon 1. Because of the infusion of FFPs before hospital admission, the patient had an MBL plasma concentration of 840 ng/mL at admission, which rapidly decreased to 290 ng/mL 72 h after admission. A total of 7 patients were heterozygous (A/O) for a mutation in exon 1 of the MBL gene and had a median MBL plasma concentration at hospital admission of 360 ng/
Figure 2. Time course of complement activation products for patients with meningococcal septic shock. Black squares indicate A/A (mannose-binding lectin [MBL] wild type) and A/O (heterozygous exon 1 mutation) patients with shock, open circles represent MBL–deficient patient 1, and open triangles represent MBL-deficient patient 2. C3bc and terminal complement complex (TCC) reflect common pathway and terminal pathway activation, respectively, C4bc reflects classical or lectin pathway activation, and C3bBbP reflects alternative pathway activation. For the A/A and A/O patients, medians values and interquartile ranges are presented.

mL (range, 30–680 ng/mL). Nine patients had a normal genotype (A/A); these patients had a median MBL plasma concentration at hospital admission of 1750 ng/mL (range, 1110–9000 ng/mL). Four patients presented without shock; 2 had a normal MBL genotype, and 2 were heterozygous for an exon 1 mutation in the MBL gene (median MBL plasma concentration at admission, 2351 ng/mL; range, 266–4350 ng/mL).

The A/A and A/O patients with shock experienced more-fulminant and more-severe disease (reflected by a higher mortality or more sequelae, a shorter time from disease onset to hospital admission, and a higher PRISM score), compared with that experienced by patients without shock. In addition, plasma concentrations of the cytokines TNF-α, IL-1β, and IL-10 were also increased in patients with shock, compared with concentrations in patients without shock. The characteristics of the patients with shock who were O/O (patient 1 and patient 2) were within the ranges of the characteristics of the other patients with shock, although the former had a relatively low disease severity.

In the A/A and A/O patients with shock, considerably higher plasma concentrations of C3bc (reflecting systemic common pathway activation) and TCC (reflecting systemic terminal pathway activation) were seen at hospital admission, compared with concentrations in O/O patients and in the patients without shock (Figure 1). C4bc (reflecting classical or lectin pathway activation) was higher in the A/A patients with shock (median concentration, 27 AU/mL; range, 8–68 AU/mL; lower quartile, 19 AU/mL) and A/O patients with shock (median concentration, 32 AU/mL; range, 15–168 AU/mL; lower quartile, 22 AU/mL) than it was in the O/O patients (16 and 22 AU/mL). No differences in activation of the classical pathway (C1rs-C1inh complexes) were seen for these patients (data not shown). C3bBbP (reflecting alternative pathway activation) was highest in the A/A patients with shock, followed by the A/O patients, and was lowest in patient 1 and patient 2 (the O/O patients) and in the patients without shock. Thus, whereas high values of systemic complement activation were found in the patients with shock who were MBL sufficient, low values of complement activation products were found in the MBL-deficient patients, indicating that MBL is of vital importance for the systemic activation of the complement system seen during MSS.

Time-course analysis showed that markedly increased values of C3bc, TCC, C4bc, and C3bBbP complement activation products could be found until 8 h after hospital admission in the A/A and A/O patients with shock. Complement activation in the 2 MBL-deficient patients was consistently low during the first 48 h of disease. After 24 h, concentrations of complement activation products in the MBL-deficient patients with shock were similar to those found in the MBL-deficient patients (Figure 2) and in the patients without shock (data not shown).

In the patients with septic shock, complement activation correlated significantly with disease severity (R = 0.72 as calculated with the Spearman rank test; P < .001 for the correlation between TCC and PRISM; Figure 3). In addition, high plasma
D-dimer concentration at hospital admission and low platelet count nadir (i.e., the lowest value within the first 48 h after hospital admission) [28] significantly correlated with plasma concentration of TCC (for D-dimers: R = 0.73, P < .001; for platelet count nadir: R = −0.88, P < .001) (Figure 3). In addition, TCC concentrations were statistically significantly higher in the patients with shock who had overt DIC (DIC score, ≥5), compared with TCC concentrations in the patients without DIC (median TCC concentration, 5 AU/mL vs 2.77 AU/mL; P = .006). This indicates that systemic activation of complement is closely related to the development of DIC.

Additional in vitro experiments with convalescent blood samples obtained from one of the MBL-deficient patients (patient 1) and from an age-matched control subject who had experienced meningococcal meningitis were performed. Patient 1 had normal classical and alternative pathway hemolytic activity (CH50, 104% [normal range, 67%–133%]; AP50, 87% [normal range, 67%–133%]). In a whole blood model of meningococcal sepsis, significant C4bc and C3bBbP activation was seen in the control patient, whereas no lectin pathway activation and markedly reduced alternative pathway activation was found in the MBL-deficient patient (Figure 4). Similarly, substantial total complement activation, reflected by formation of C3bc and TCC, was found in the control patient but was largely absent in the MBL-deficient patient (Figure 4).

**DISCUSSION**

This is the first report describing complement activation during MSS in relation to genotype for MBL. Substantially higher plasma concentrations of the complement activation products C3bc, TCC, C4bc, and C3bBbP were seen in the A/A and A/O patients with MSS than in the MBL-deficient (O/O) patients and the patients without shock. This indicates that there is an important role for MBL in the systemic activation of complement during MSS. In addition, we found that systemic complement activation was closely related to the development of DIC.

The mechanism of complement activation during MSS has been debated, and classical as well as alternative pathway–dependent routes of activation have been proposed [5, 29]. However, these studies were performed before the contribution of the lectin pathway could be assessed. We have previously found that complement activation in a whole blood model of meningococcal sepsis is inhibited by anti-MBL antibodies, indicating that, in whole blood, complement activation by meningococci is, at least in part, dependent on MBL [8]. The present in vivo results support the previous in vitro findings. In addition, by using convalescent blood samples obtained from one of the patients who was homozygous for a mutation in exon 1 of the MBL gene, we confirmed in vitro that MBL deficiency leads to the absence of lectin pathway activation and decreased common pathway activation, compared with that found in an MBL-sufficient control patient.
The basis of our conclusion that MBL contributes to complement activation during MSS is based on our observations regarding 2 MBL-deficient patients. Although this is a low number of patients, we think that our conclusions are valid, not only because we can replicate our findings in 2 patients and our time course analysis implicates that complement activation in MBL-deficient patients is consistently low during the first 48 h of disease, but also because our results perfectly fit with the in vitro results published earlier and other reports on MBL consumption during gram-negative sepsis [8, 30].

One of the MBL-deficient patients (patient 2) had a plasma concentration of MBL at hospital admission that was similar to that found in heterozygous patients, which was consistent with the transfusion of large amounts of FFP before hospital admission. We have also observed this phenomenon in other patients who were given FFP and were heterozygous for an exon 1 mutation. For example, in 1 patient, an MBL plasma concentration at hospital admission of 495 ng/mL increased to 2160 ng/mL after FFP transfusion and decreased again to 1135 ng/mL (at 48 h after admission) and 915 ng/mL (at 144 h after admission). In addition, this phenomenon has also been observed in MBL-deficient patients who were given FFPs after cardiac surgery [31]. Interestingly, the infusion of FFP in patient 2 did not lead to measurable complement activation. We suggest that this might be attributable to a lack of complement activation during the disease course before FFP was given, combined with a rapid clearance of meningococci and complement activation intermediates after the start of treatment.

During MSS and in the whole blood model, the alternative pathway was considerably activated in MBL-sufficient patients but was reduced in the patients with MBL deficiency. This is consistent with the view that the role of the alternative pathway lies in amplification of complement activation; in this case, through MBL initiated lectin pathway activation [8, 32].

Heterozygosity for an exon 1 mutation in the MBL gene had a limited effect on complement activation during meningococcal septic shock, although median values of C3bc, TCC, and C3bBbP were somewhat lower in this group, compared with the A/A group. This suggests that MBL-dependent complement activation is sufficiently maintained in plasma with reduced MBL levels, as in A/O genotypes, but is strongly affected when it falls below a certain limit and is lost in O/O plasma [33]. Three A/O patients had MBL plasma concentrations below 200 ng/mL; these very low concentrations may be related to consumption of MBL during septic shock, reflecting the binding of MBL to microbes to activate complement [30].

The 2 MBL-deficient patients had a relatively low disease severity. This parallels the findings regarding patients with terminal complement component deficiencies [3] and is in line with the study by Hibberd et al [10], which showed that, although susceptibility to meningococcal disease was increased by MBL deficiency, disease severity seemed to be reduced. In contrast, patients with alternative pathway deficiencies (properdin or factor D) who also have increased susceptibility to meningococcal disease are reported to have a more fulminating disease course [3, 34, 35]. Thus, it seems that, whereas MBL has a protective effect for susceptibility to meningococcal disease, MBL deficiency during septic shock may be associated with lower disease severity. Apparently, there is a slender balance between susceptibility to meningococcal infection and the course of disease.

Crucial for the development of tissue damage and limb-ischemia in MSS is the severity of DIC. Experimentally, C5a is a potent inducer of tissue factor expression, which is critical in the development of DIC [36–38]. In addition, TCCs induce secretion of large von Willebrand factor multimers [39], which may contribute to the thrombopenia seen during DIC. We found statistically significantly higher plasma concentrations of TCC in those patients with overt DIC, and the degree of systemic complement activation was strongly correlated with DIC parameters. In the MBL-deficient patients, platelet counts and fibrinogen concentrations were relatively preserved, suggesting that these patients also had less severe coagulopathy, although 1 of the MBL deficient patients had overt DIC (as defined by a DIC score of ≥5). This may indicate that MBL-dependent complement activation, which leads to the formation of C5a, is important for the development of DIC.

Activation of the cytokine network is considered to be one of the main pathological events during MSS [1]. The extent of the inflammatory cytokine response in the MBL-deficient patients was similar to that of the other patients with shock. This indicates that the lower disease severity and the absence of DIC in the MBL-deficient patients, compared with the other patients, was not caused by a reduction in cytokine activation.

To date, no adjunctive immunomodulatory treatment is available that is proven to influence the course of disease in MSS. Modulation of the complement system is postulated to be an attractive approach, based on in vitro research and data in animal experiments [8, 40, 41]. New therapies that aim to modulate the complement system during MSS should at least also target the effects of MBL-dependent complement activation.

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