CHAPTER 2

Molecular and immunological characteristics of MBL
CHAPTER 2.1

Functional characterization of the lectin pathway of complement in human serum

Anja Roos, Lee H. Bouwman, Jeric Munoz, Tahlita Zuiverloon, Maria C. Faber-Krol, Francien C. Fallaux-van den Houten, Ngaisah Klar-Mohamad, C. Erik Hack, Marcel G. Tilanus, and Mohamed R. Daha

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ABSTRACT

Mannan-binding lectin (MBL) is a major initiator of the lectin pathway (LP) of complement. Polymorphisms in exon 1 of the MBL gene are associated with impaired MBL function and infections. Functional assays to assess the activity of the classical pathway (CP) and the alternative pathway of complement in serum are broadly used in patient diagnostics. We have now developed a functional LP assay that enables the specific quantification of autologous MBL-dependent complement activation in human serum.

Complement activation was assessed by ELISA using coated mannan to assess the LP and coated IgM to assess the CP. Normal human serum contains IgG, IgA and IgM antibodies against mannan, as shown by ELISA. These antibodies are likely to induce CP activation. Using C1q-blocking and MBL-blocking mAb, it was confirmed that both the LP and the CP contribute to complement activation by mannan. In order to quantify LP activity without interference of the CP, LP activity was measured in serum in the presence of C1q-blocking Ab. Activation of serum on coated IgM via the CP resulted in a dose-dependent deposition of C1q, C4, C3, and C5b-9. This activation and subsequent complement deposition was completely inhibited by the C1q-blocking mAb 2204 and by polyclonal Fab anti-C1q Ab. Evaluation of the LP in the presence of mAb 2204 showed a strong dose-dependent deposition of C4, C3, and C5b-9 using serum from MBL-wildtype (AA) but not MBL-mutant donors (AB or BB genotype), indicating that complement activation under these conditions is MBL-dependent and C1q-independent. Donors with different MBL genotypes were identified using a newly developed oligonucleotide ligation assay for detection of MBL exon 1 polymorphisms.

We describe a novel functional assay that enables quantification of autologous complement activation via the LP in full human serum up to the formation of the membrane attack complex. This assay offers novel possibilities for patient diagnostics as well as for the study of disease association with the LP.
Activation of the complement system is an important component of host defense. Following infection, triggering of the complement activation cascade via direct binding of complement components to microbial surfaces may lead to opsonization and pathogen elimination via humoral and cellular mechanisms. Furthermore, complement activation may trigger and amplify the acquired immune system. Until now, three different pathways of complement activation have been described, i.e. the classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the level of C3, leading to activation of the common terminal complement pathway and finally formation of the membrane attack complex (1, 2).

Defects in the complement system may lead to a partial or complete blockade of the complement activation cascade. Depending on the level of the defect, either the induction phase or the effector phase of complement activation may be hampered, and the defect may affect more than one pathway. Impaired function of the complement system may occur due to genetic defects, or due to acquired deficiencies of complement components. Acquired complement deficiencies may occur due to formation of autoantibodies to complement components or due to excessive complement consumption (1-3). Genetic complement deficiencies have been described at all levels of the system, i.e., in the classical pathway, in the alternative pathway, in the lectin pathway, and in the terminal pathway from C3 to C9 (4).

Most complement defects are associated with disease, ranging from a relatively mild increase in the susceptibility to infections to the occurrence of a severe systemic autoimmune syndrome. Furthermore, impaired complement function is associated with the occurrence of flares in patients with systemic lupus erythematosus (SLE) (1, 2, 4). Therefore, functional assays to measure complement activity in human serum have a clear diagnostic and prognostic value.

Complement function in serum is mostly measured using hemolytic assays that enable the functional assessment of the classical complement pathway and the alternative complement pathway, respectively. In these hemolytic assays, the function of the complement pathways is expressed as its ability to generate the C5b-9 complex upon activation. However, such an assay is currently not available for the evaluation of the lectin pathway of complement in serum.

The lectin pathway of complement (LP) is mainly driven by binding of mannan-binding lectin (MBL) to one of its carbohydrate ligands (5). Binding will induce activation of the MBL-associated serine proteases (MASP) leading to formation of the C3 convertase C4b2a (6-8). The MBL-MASP complex, being the recognition complex of the LP, has a strong structural and functional similarity to the C1 complex, the recognition unit of the classical complement pathway (CP). The C1 complex, consist-
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The gene encoding human MBL is characterized by a high degree of polymorphisms, both in the promoter region and in exon 1 (9). In the promoter region, various single nucleotide polymorphisms (SNP) have been described that are involved in quantitative gene expression and hence determine the MBL plasma concentration. Furthermore, at least five different SNPs have been discovered in exon 1 of the MBL gene, encoding the collagenous region of MBL (10-13). At codon 52 (D genotype), codon 54 (B genotype) and codon 57 (C genotype), SNPs are frequently present: the allele frequency in the Caucasian population is 5% (D allele), 13% (B allele) and 2% (C allele), respectively (9). These SNPs induce amino acid substitutions that affect the polymerization of the MBL molecule in a dominant way. Accordingly, small-sized MBL molecules are generated with impaired functional properties (14-16).

The presence of MBL-mutant alleles is associated with increased susceptibility to infections, mainly in childhood and in immune-compromised individuals (17-20). Furthermore, the above-described SNPs confer an increased progression of severe chronic diseases such as cystic fibrosis, rheumatoid arthritis, and SLE (21-23). Therefore, since there is such a high inter-individual variability in expression of (functional) MBL, which is determined by multiple variables, functional assessment of LP activity in human serum generates novel and most likely clinically more relevant possibilities for risk assessment for individual patients.

We have now developed an ELISA-based LP assay that enables the functional evaluation of successive steps of autologous complement activation in full human serum without any interference of the CP. Measurement of the activity of the CP and the alternative complement pathway (AP) in a similar ELISA system provides the possibility of parallel quantification of all three complement activation pathways in patient serum using one assay system.

**MATERIALS AND METHODS**

**Human materials**

Human serum was obtained from 70 healthy adult volunteers and immediately frozen at -80°C in aliquots. Genomic DNA was isolated from heparinized blood as described below. Human DNA samples with known MBL gene polymorphisms were kindly provided by Dr. P. Garred, (Copenhagen, Denmark). Outdated healthy donor plasma was obtained from the Bloodbank Leiden-Haaglanden, Leiden, the Nether-
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lands. From a patient with Waldenström’s macroglobulinemia, plasma was obtained that became available after a plasmapheresis treatment.

**Anti-C1q and anti-MBL antibodies**

Monoclonal antibodies directed against C1q were produced in mice as described before (24). The anti-C1q mAb 2204 (IgG1) is directed against the globular head domain of C1q and is able to inhibit the binding of C1q to IgG, as well as C1q-dependent hemolysis (25). For the purification of mAb 2204, gamma globulins were precipitated from ascites using 50% (NH₄)₂SO₄. The precipitate was dialyzed against 10 mM Tris containing 2 mM EDTA (pH 7.8) and subjected to anion exchange chromatography using DEAE-Sephacel (Pharmacia, Uppsala, Sweden). Proteins were eluted using a salt gradient and the fractions that showed binding of mouse IgG to C1q-coated ELISA plates in the presence of 1 M NaCl were pooled, concentrated, dialyzed against PBS and stored at -80°C.

Polyclonal anti-C1q antibodies were produced in rabbits. New Zealand White rabbits were immunized (weekly for four weeks) with 180 µg C1q dissolved in complete Freunds adjuvant, resulting in antisera with a positive titer on C1q-coated ELISA plates beyond 1/25,000. IgG was precipitated from rabbit serum using 40% (NH₄)₂SO₄ and purified using DEAE-Sephacel as described above.

Starting from purified rabbit IgG anti-C1q, Fab fragments were generated using papain. IgG was dialyzed against 10 mM phosphate buffer containing 10 mM L-cysteine and 2 mM EDTA (pH 7.0). Subsequently, mercuripapaine (from Sigma, St. Louis, MO) was added (1% w/w of the protein content) followed by incubation for 16 hours at 37°C. After dialysis against PBS, the sample was applied to Sepharose-coupled protein G (from Pharmacia), and the fall through fractions, containing Fab fragments, were pooled, concentrated, and used for experiments. Analysis by non-reducing SDS-PAGE showed a prominent band at approximately 45 kD.

A mouse mAb directed against the lectin domain of human MBL (mAb 3F8) was kindly provided by Dr. G.L. Stahl (Harvard Medical School, Boston, Massachusetts, USA) (26).

**Preparation of human C1q and C1q-depleted serum**

Human C1q was isolated from human donor plasma exactly as described previously and was stored at -80°C (25). Isolated C1q was able to completely restore the lysis of antibody-coated erythrocytes in the presence of C1q-depleted human serum.

For the preparation of C1q-depleted serum, undiluted normal human EDTA-plasma (obtained from a donor with the MBL/AA genotype) was applied on column consisting of rabbit IgG anti-human C1q coupled to Biogel A5 (from Biorad). The column was washed using Veronal-buffered saline (VBS; 1.8 mM Na-5,5-diethylbarbital,
0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10 mM EDTA. Fractions were tested in a C1q-dependent hemolytic assay in the absence or presence of purified C1q, as previously described (25). Fractions that showed complete erythrocyte lysis in the presence of C1q, but not in the absence of C1q, were pooled and concentrated until the original volume. After recalcification, C1q-depleted serum was stored at -80°C.

Isolation of human IgM

Plasma containing an IgM paraprotein was dialyzed against 10 mM sodium acetate containing 2 mM EDTA (pH 5.0). The precipitated proteins were recovered by centrifugation, dissolved in PBS, dialyzed against Tris/EDTA buffer (10 mM Tris, 2 mM EDTA, pH 7.8 and conductivity 5.0 mS), and subjected to anion exchange chromatography using DEAE-Sephacel. IgM that eluted in the salt gradient was pooled, dialyzed against 10 mM sodium acetate (6.0 mS, pH 7.0) and applied to a CM-C-50 Sephadex anionic exchange column (from Pharmacia). Following elution with a salt gradient, fractions containing IgM were pooled, concentrated, and applied to a Superdex 300 gel filtration column. Peak fractions containing IgM and free of IgG were pooled, concentrated, and stored at -80°C.

Assessment of functional lectin pathway activity by ELISA

Functional activity of the lectin pathway was assessed by ELISA using immobilized mannan as a ligand. Mannan was obtained from Sigma (from Saccharomyces Cerevisiae; M7504), dissolved in PBS (10 mg/ml) and stored at -20°C. Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with mannan (100 µg/ml; 100 µl per well) in coating buffer (100 mM Na₂CO₃/NaHCO₃, pH 9.6), for 16 hours at room temperature or for 2 hours at 37°C. After each step, plates were washed three times with PBS containing 0.05% Tween 20. All incubation volumes were 100 µl. Residual binding sites were blocked by incubation with PBS containing 1% BSA for one hour at 37°C. Serum samples were diluted in GVB++ (VBS containing 0.5 mM MgCl₂, 2 mM CaCl₂, 0.05% Tween-20, and 0.1% gelatin; pH 7.5) in the presence of mAb 2204 (20 µg/ml) as an inhibitor of C1q, unless otherwise indicated. This mixture was pre-incubated for 15 minutes on ice, before addition to the plates. The plates were then sequentially incubated for 1 hour at 4°C and for 1 hour at 37°C, followed by washing. Complement binding was detected using mouse mAb conjugated to digoxigenin (dig) using digoxigenin-3-O-methylcarbonyl-α-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of MBL, C1q, C4, C3, and C5b-9 was performed using mAb 3E7 (anti-human MBL, kindly provided by Dr. T. Fujita, Fukushima, Japan), mAb 2214 (anti-human C1q), mAb C4-4a (anti-human C4d), RFK22
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(anti-human C3) and AE11 (anti-human C5b-9, kindly provided by Dr. T.E. Mollnes, Oslo, Norway), respectively. Binding of mAb was detected using dig-conjugated sheep anti-mouse antibodies (Fab fragments) followed by HRP-conjugated sheep anti-dig antibodies (Fab fragments, both from Boehringer Mannheim). All detection antibodies were diluted in PBS containing 1% BSA and 0.05% Tween 20. Enzyme activity of HRP was detected following incubation with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (from Sigma; 2.5 mg/ml in 0.1 M Citrate/Na$_2$HPO$_4$ buffer, pH 4.2) in the presence of 0.01% H$_2$O$_2$, for 30-60 min. at room temperature. The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).

**Assessment of functional classical pathway activity by ELISA**

The protocol for the functional activity of the classical pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for CP activation, human IgM was coated at 2 µg/ml. After blocking of residual binding sites, serum samples, diluted in GVB++, were added to the plate and incubated for 1 hour at 37°C. Complement binding was assessed using dig-conjugated mAb directed against C1q, C4, C3, and C5b-9, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

**Assessment of functional alternative pathway activity by ELISA**

The protocol for the functional activity of the alternative pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for AP activation, LPS was coated at 10 µg/ml. LPS from Salmonella Typhosa was obtained from Sigma (L-6386), dissolved in PBS at 1.6 mg/ml and stored at -20°C. Plates were blocked using 1% BSA in PBS. Serum samples were diluted in GVB/MgEGTA (VBS containing 10 mM EGTA, 5 mM MgCl$_2$, 0.05% Tween-20, and 0.1% gelatin; pH 7.5) and incubated in the plate for 1 hour at 37°C. Complement binding was assessed using dig-conjugated mAb directed against C4 and C3, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

**Quantification of anti-mannan antibodies in human serum**

For the quantification of anti-mannan antibodies in human serum, ELISA plates were coated with mannan and blocked with 1% BSA in PBS. Serum samples were diluted 1/100 for detection of IgG anti-mannan Ab, 1/10 for detection of IgA anti-mannan Ab, and 1/40 for detection of IgM anti-mannan Ab, respectively, unless otherwise indicated. For quantification, pooled human IgG (48 mg/ml IgG), pooled human IgA (41 mg/ml IgA), and pooled human IgM (35 mg/ml IgM) were used as a standard for detection of IgG, IgA and IgM anti-mannan antibodies, respectively (kindly provided...
by Biotest Pharma GmbH, Dreieich, Germany). The concentration of anti-mannan antibodies in these preparations was arbitrarily set at 1000 U/ml. All samples were diluted in PBS containing 0.05% Tween 20 and 1% BSA. Antibody binding was detected using biotinylated HB43 (mouse mAb anti-human IgG), biotinylated HB57 (mouse mAb anti-human IgM) and dig-conjugated 4E8 (mouse mAb anti-human IgA), respectively, followed by either HRP-conjugated streptavidin or HRP-conjugated sheep anti-dig antibodies (both from Boehringer).

DNA isolation

Genomic DNA was isolated from heparinized blood according to standard procedures (27). Briefly, 10 ml blood was diluted with 40 ml EL buffer (erythrocyte lysis buffer: 155 mM NH$_4$Cl, 10 mM KHCO$_3$, 1 mM EDTA, pH 7.4) and incubated on ice for 20 minutes. After centrifugation (10 minutes at 500 g), the pellet was washed with 25 ml EL buffer, and resuspended in 3 ml of KL buffer (10 mM Tris, 2mM EDTA, 400 mM NaCl, pH 8.4), followed by thoroughly shaking. After addition of 25 μl pronase (20 mg/ml in water, Boehringer Mannheim, Germany) and 150 μl SDS (20% in water), the mixture was incubated in a shaking water bath at 37°C for 18 hours. Finally, the DNA was precipitated with ethanol, dissolved in 0.5 ml TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4), heated for 5 minutes at 65°C, and kept at 4°C.

PCR amplification of exon 1 of the MBL gene

Exon 1 of the MBL gene was amplified from genomic DNA by PCR. Starting from 1 μl of genomic DNA (approximately 0.7 μg), a 40 μl PCR reaction was performed, using 0.25 mM dNTP (from Pharmacia Biotech), 0.8 U Amplitaq (from Perkin Elmer,

<table>
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<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>PCR forward</td>
<td>5'-ACCCAGATTGTAGGACAGAG-3'</td>
</tr>
<tr>
<td>PCR reverse</td>
<td>5'-GGTTGTTTCTCCTGTCACAG-3'</td>
</tr>
<tr>
<td>OLA 52-common</td>
<td>5'-P-CCCATCTTTGCCTGG-bio-3'</td>
</tr>
<tr>
<td>OLA 52-wildtype</td>
<td>5'-dig-CCCTGTTGCCCATCACG-OH-3'</td>
</tr>
<tr>
<td>OLA 52-mutant</td>
<td>5'-dig-CCCTGTTGCCCATCAG-OH-3'</td>
</tr>
<tr>
<td>OLA 54-common</td>
<td>5'-P-CATACGCCTCATCTGG-bio-3'</td>
</tr>
<tr>
<td>OLA 54-wildtype</td>
<td>5'-dig-CCCTTCTCCCTGTTGC-OH-3'</td>
</tr>
<tr>
<td>OLA 54-mutant</td>
<td>5'-dig-CCCTTCTCCCTGTTG-OH-3'</td>
</tr>
<tr>
<td>OLA 57-common</td>
<td>5'-P-CCCTGTTGCCCATCAG-bio-3'</td>
</tr>
<tr>
<td>OLA 57-wildtype</td>
<td>5'-dig-TGTTTCCCCCTTCTT-OH-3'</td>
</tr>
<tr>
<td>OLA 57-mutant</td>
<td>5'-dig-TGTTTCCCCCTTCTT-OH-3'</td>
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PCR primers were derived from (9) with slight modifications. OLA primers are labelled with either biotin (bio) or digoxigenin (dig). All primers were obtained from Eurogentec (Seraing, Belgium).
Wellesley, MA), and 12.5 pmol of both PCR primers (from Eurogentec, Seraing, Belgium; Table 1) in PCR buffer (10 mM Tris HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.6 mg/ml BSA, pH 8.3). The PCR reaction was performed in a Peltier Thermal Cycler (PTC200, from MJ Research, Waltham, MA) using the following program: denaturation for 5 min at 95°C, followed by 36 cycles of 1 min. 95°C, 1 min. 57°C, and 1 min. 72°C, and a final elongation period for 7 min. at 72°C. Evaluation of the PCR products by agarose electrophoresis showed one specific band of the expected molecular weight (679 bp) with an estimated concentration of about 30 ng/µl.

Oligonucleotide Ligation Assay (OLA) for MBL genotyping

For detection of MBL mutant alleles at codon 52, 54, and 57, three different OLA protocols were developed. For each OLA, two reactions were performed in parallel, using either the wildtype or the mutant primer, both in combination with a common primer (Table 1). PCR products were first heated for 5 min at 99°C. The OLA reaction was performed in a 20 µl reaction mixture consisting of 2 µl of PCR product, 5 pmol common primer, 5 pmol of either the wildtype or the mutant primer (Table 1), and 1.2 U Taq DNA ligase, using the buffer supplied by the manufacturer (from New England Biolabs, Beverly, MA). The following program was run in a PTC 200 Thermal Cycler: denaturation for 2 min. at 94°C, 10 cycles of 10 sec. 94°C and 3 min. 60°C, followed by a final incubation of 5 min. at 99°C. For OLA detection of codon 57 polymorphisms, probe anealing was performed at 54°C instead of 60°C.

For detection of OLA products, ELISA plates were coated with avidin (20 µg/ml, from ICN Biomedicals inc., Aurora, Ohio, USA) and aspecific binding sites were blocked with PBS containing 3% BSA. The OLA reaction mixture was 1/5 diluted in PBS containing 1% BSA, added to the plate, and incubated for 1 hour at 37°C. Plates were washed and dig-conjugated reaction products were detected using HRP-conjugated sheep anti-dig antibodies as described above.

RESULTS

Anti-mannan antibodies in human serum

Mannan is a major ligand of MBL that can efficiently activate the LP of complement. Human serum contains anti-carbohydrate antibodies, probably resulting from previous microbial contacts. Such anti-carbohydrate antibodies may bind to mannan and the resulting immune complex may contribute to complement activation by mannan via activation of the classical complement pathway (28; 29). Mannan-binding antibodies are clearly detectable in human serum as assessed by ELISA (Fig. 1). Incubation of pooled human IgG (Fig. 1A), IgA (Fig. 1B) and IgM (Fig. 1C) on
immobilized mannan resulted in a dose-dependent binding of IgG, IgA, and IgM as detected by isotype-specific mAb. As a control, parallel incubations were performed on immobilized BSA, resulting in low or undetectable background binding of pooled Ig. Incubation of three sera from healthy donors on mannan-coated plates resulted in strong dose-dependent IgG binding in all three sera. In donor 1, IgA and IgM anti-mannan Ab were undetectable, serum from donor 2 contained IgG, IgA and IgM anti-mannan antibodies, whereas in donor 3 some IgM binding was observed but no IgA binding (Fig. 1A-C). Binding of Ig was undetectable following incubation of serum on BSA-coated plates (Fig. 1A-C). Quantification of anti-mannan antibodies in serum from 70 healthy donors is presented in fig. 1D. IgG and IgM anti-mannan Ab were present in nearly all donors, with a large interindividual variation, whereas IgA anti-mannan Ab were detected in 63% of the donors. No significant correlation was observed between the three major isotypes of anti-mannan antibodies, or between anti-mannan antibodies and MBL concentrations (not shown).
Functional characterization of the lectin pathway in the presence of C1q-inhibitory Ab

Both the LP and the CP are calcium-dependent and lead to activation of C4. A distinction between both pathways can be made by selection of a specific ligand that induces activation of either the LP or the CP. In view of the presence of anti-mannan Ab in human serum, mannan is likely to activate both the LP, via MBL, and the CP, via anti-mannan Ab. Therefore, a strategy was developed to inhibit activation of the CP in order to allow solely the activation of the LP by immobilized mannan, by using inhibitory anti-C1q antibodies.

Anti-C1q antibodies were tested for their ability to inhibit the CP of complement using immobilized IgM as a specific activator of the CP. Incubation of 1% normal human serum (NHS) on immobilized IgM induces deposition of C4, which could be dose-dependently inhibited by the anti-C1q mAb 2204, by rabbit IgG anti-C1q antibodies and by Fab fragments prepared from this rabbit anti-C1q antibody preparation (Fig. 2A). Complete inhibition was reached when the antibodies were applied at 5 µg/ml. In contrast, rabbit IgG prepared from non-immunized rabbits did not have an effect on C4 activation via the CP. These antibodies were tested for their effect on complement activation induced by immobilized mannan. Incubation of NHS on mannan induced a dose-dependent deposition of C4, with a maximal activation at a serum concentration of 1% (Fig. 2B). Addition of a fixed concentration of mAb 2204, Fab anti-C1q fragments, or normal rabbit IgG as a control had a slight inhibitory effect on C4 activation. In contrast, rabbit IgG anti-C1q Ab induced complete inhibition of C4 activation by mannan, most likely due to complement consumption via C1q-anti-C1q complexes (Fig. 2B). These data show that C1q-inhibitory antibodies can block CP activation completely whereas mannan-induced activation of the LP can proceed in a C1q-independent way.

To further examine the role of C1q in complement activation by mannan and by IgM, NHS was depleted of C1q. Depletion of C1q from NHS resulted in a complete inhibition of C4 activation by immobilized IgM (Fig. 3A), as previously described (30), whereas C4 activation by immobilized mannan was slightly inhibited by depletion of C1q (Fig. 3B). Reconstitution of C1q-depleted serum with purified C1q resulted in a complete restoration of C4 activation by IgM (Fig. 3C). In contrast, C4 activation by mannan was slightly inhibited by addition of purified C1q to C1q-depleted serum, possibly due to the presence of an inhibitory protein co-isolated with C1q. The contribution of C1q and MBL to C4 activation by IgM and mannan was further studied using blocking mAb against C1q and MBL, respectively (Fig. 3D). C4 activation on IgM-coated plates was completely inhibited by mAb anti-C1q and no inhibition occurred with a blocking anti-MBL mAb. In contrast, C4 activation induced by mannan was partially inhibited by mAb anti-C1q and strongly inhibited by mAb anti-MBL. Complete inhibition of mannan-induced C4 activation was achieved when
a combination of mAb anti-C1q and mAb anti-MBL was used (Fig. 3D). Together, these data indicate that IgM-mediated activation of C4 is completely dependent on C1q and does not involve MBL. In contrast, mannan-induced activation of C4 is mainly mediated by the LP but comprises a minor contribution of the CP. The latter contribution of the CP can be inhibited by C1q-blocking Ab, thus allowing activation of the LP only.

**Complement activation and formation of C5b-9 via the CP and via the LP**

The complement activation cascade was further studied using mAb to detect binding of specific complement components upon their activation via the CP and the LP, respectively. Incubation of NHS on immobilized IgM resulted in a dose-dependent
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deposition of C1q, C4, C3, and C5b-9 to the plate (Fig. 4A). Binding of C1q and subsequent complement activation induced by IgM could be completely inhibited by mAb 2204. Incubation of NHS on immobilized mannan resulted in dose-dependent binding of C4, C3 and C5b-9, whereas binding of C1q was hardly detectable (Fig. 4B). Complement activation by mannan was only slightly inhibited by addition of mAb 2204. Therefore, addition of mAb 2204 in serum allows the specific detection of LP activation using mannan as a ligand, without interference of the CP.

Activation of the alternative pathway

To enable the detection of all complement activation pathways in one assay system, we also studied activation of the alternative pathway in an ELISA system. In contrast to the LP and the CP, activation of the AP is calcium-independent. Therefore, a calcium-free buffer was used, thus excluding involvement of the CP and the LP. As previously described (30), incubation of NHS in a buffer containing EGTA and Mg²⁺ on plates coated with LPS resulted in a dose-dependent deposition of C3 (Fig. 5A).
Figure 4. Complement activation via the LP and the CP. Complement activation was induced by incubation of different concentrations of NHS on plates coated with IgM for CP activation (A) or with mannan for LP activation (B), in the presence or absence of mAb 2204 (20 µg/ml). Activation and binding of complement was demonstrated by detection of C1q, C4, C3, and C5b-9 using specific mAb. Results represent mean ± SD from one out of at least three experiments.
Some activation of C3 was also observed on plates coated with BSA only. Surprisingly, strong activation of C3 was also observed when NHS was incubated on mannan-coated plates using the same conditions, suggesting that mannan may also support activation of the AP (Fig. 5A). Detection of C3 was reduced until background levels when EDTA was present in the complement source (not shown). As expected from an AP-dependent mechanism, C3 activation in calcium-free buffers required a serum concentration that is about 10-fold higher than that required for C3 activation by mannan in a calcium-containing buffer via the LP (compare fig. 5A with fig. 4B).

Although C3 activation was clearly detectable in a calcium-free buffer, no activation of C4 could be established (Fig. 5B), suggesting that under these conditions activation of C3 is independent of MBL binding and C4 activation.

**MBL genotyping by oligonucleotide ligation assay**

Single nucleotide polymorphisms in exon 1 of the MBL gene are the most important genetic modifiers of MBL function. We developed three oligonucleotide ligation assays (OLA) for the detection of MBL exon 1 SNPs at codon 52, codon 54, and codon 57, respectively. Using this technique, the presence of B (codon 54), C (codon 57), and D alleles (codon 52), as indicated by formation of double-labeled DNA products using the mutant oligonucleotides, can be easily detected with a standard laboratory equipment, both in homozygous and in heterozygous patterns (Fig. 6).

**Lectin pathway activation is dependent on the MBL genotype**

In the Caucasian population, the B allele is the most frequent exon 1 polymorphism in the MBL gene. It has been previously reported that recombinant MBL with the BB genotype has a strongly reduced ability to support complement activation (14).
Using MBL genotyping by OLA, sera from individuals with different MBL genotypes were identified. We compared activity of the CP and the LP in serum obtained from an MBL wildtype donor (AA genotype) with serum from donors with a heterozygous
and a homozygous mutation at codon 54 (AB and BB genotype, respectively). Serum from all three donors showed binding of C1q and strong activation of C4, C3 and C5b-9 via the CP upon incubation on immobilized IgM, in a similar dose-response relationship (Fig. 7A). Upon serum incubation on immobilized mannan, strong dose-dependent binding of MBL to mannan was observed in AA serum, whereas MBL binding in AB serum was about 8-fold less and no binding of MBL to mannan could be established in BB serum (Fig. 7B, upper panel). In parallel, LP activity was assessed in the same sera by their incubation on immobilized mannan, in the presence of mAb 2204 to block the CP. In sharp contrast to the results obtained on coated IgM, only AA serum, but not BB serum nor AB serum, was able to induce detectable activation of C4, C3 and C5b-9 via the LP (Fig. 7B). These results indicate that LP activity is dependent on the presence of functionally active MBL.

**DISCUSSION**

In the present study, we describe a novel assay for the detection of functional activity of the LP of complement. The assay is based on the detection of various stages of complement activation induced by binding of MBL to immobilized mannan, and involves the addition of inhibitory anti-C1q antibodies to prevent interference of activation of the CP. We demonstrate that in this novel assay system activation of autologous C4, C3, and C5b-9 in full human serum is totally dependent on the presence of functionally active MBL.

Our results show the broad presence of anti-mannan antibodies in the human population. These antibodies may be produced in response to a previous yeast contact and/or may belong to the so-called natural antibodies. Increased levels of antibodies binding to mannan from Saccharomyces Cerevisiae have been described in patients with inflammatory bowel disease (31; 32). Certain anti-carbohydrate antibodies can be present in extremely high levels, as is the case for antibodies directed against the major xenoantigen Galα1-3Gal (33). IgG and IgM anti-carbohydrate antibodies can activate the classical complement pathway, and this mechanism is likely to contribute to anti-microbial defense, in addition to lectin-mediated mechanisms. Indeed it has been described that IgG anti-mannan antibodies contribute to opsonization of Candida albicans with C3 (34). Such a mechanism may especially be important in cases where the function of the lectin pathway of complement is impaired. In our study, we were not able to detect any significant difference in levels of anti-mannan Ab between MBL-wildtype and MBL-mutant individuals (not shown).

The presence of highly variable amounts of anti-mannan Ab in human serum necessitates a special strategy to prevent involvement of CP activity in mannan-based
Figure 7. Lectin pathway activity is impaired in serum from MBL-mutant donors. Serum was obtained from three different donors with a wildtype MBL genotype (AA), a heterozygous mutation (AB) or a homozygous mutation (BB) at codon 54, respectively. Different concentrations were applied to plates coated with IgM to assess CP activity, in the absence of mAb 2204 (A), or with mannan to assess LP activity, in the presence of mAb 2204 (B). Deposition of C4, C3, and C5b-9 was assessed. Results represent mean ± SD from one out of at least two similar experiments.
assays for LP activation. The present study therefore includes a specific inhibitor of C1q in the assay, which prevents any activation of the CP in serum. Until now, at least two other groups reported a functional assay for the MBL pathway activity that excluded the interference of the CP. Petersen et al. reported an elegant assay that detects the functional activity of the MBL-MASP complex in serum (28). This assay is based on the difference between the C1 complex and the MBL-MASP complex with respect to its sensitivity to ionic strength. By addition of 1 M NaCl to the serum dilution buffer, C1q binding and CP activation can be completely prevented whereas MBL binding can proceed. In the assay described by Petersen et al. (28), the serum incubation step is performed at 4 ºC, thus allowing binding of the MBL-MASP complex but not the subsequent complement activation. Activity of the complex is subsequently assessed by addition of exogenous purified C4. The advantage of this technique is that activity of the MBL-MASP complex is directly detected, without any interference of other variables in donor serum. The major difference with the technique as described in the present study is that we now describe a LP assay that assesses activation of autologous complement, which is more representative for the in vivo situation. Furthermore, our assay enables the detection of the complete complement activation cascade, up to the formation of the membrane attack complex. In this respect, our assay is comparable to hemolytic assays (CH50, AP50) generally used in clinical practice for the evaluation of CP and AP activity. The functional analysis of all three complement activation pathways in parallel by ELISA, as described in the present study, is potentially useful in routine diagnostic laboratories for a more complete diagnostic evaluation of complement defects.

An alternative functional MBL pathway assay was recently described by Zimmermann-Nielsen et al. (35). This assay also includes 1 M NaCl in the incubation buffer, but analyzes autologous C4 activation. However, activation of C4 in the presence of 1M NaCl is highly inefficient (28) and our own unpublished observations), which is apparent from the low serum dilutions used in this study. These suboptimal conditions may have a differential effect on C4 activation in serum from various donors, and therefore the C4 activation assessed in this respect is difficult to interpret. Furthermore, also in this assay it is not possible to assess complement activation at a later stage than C4, since formation of C4b2a is strongly dependent on ionic strength (36), and accordingly C3 activation is undetectable in 1 M NaCl (our unpublished observations).

An alternative approach for the analysis of LP activity in serum is the quantification of serum-induced hemolysis of mannan-coated sheep erythrocytes (6; 37). MBL is able to bind to these coated erythrocytes (37), leading to complement activation and erythrocyte lysis. Also in such an hemolytic assay, it is important to prevent interference of the classical complement pathway, which may occur both by anti-mannan
antibodies and by anti-erythrocyte antibodies. Suankratay et al. described a method in which mannan-coated erythrocytes were pre-sensitized with purified MBL, followed by incubation with serum in the presence of MgEGTA (37). This hemolytic assay analyzes the activity of the lectin pathway of complement most likely from C4 until C9, and hence does not provide information about the activity of the MBL-MASP complex in the serum source. Therefore, this assay can not be used to detect a functional impairment of LP activity at the level of MBL. We did not succeed to set up an hemolytic assay with mannan-coated erythrocytes using full serum and a C1q inhibitor, probably due to insufficient sensitivity (data not shown).

We show in the present study that both C1q and MBL have a contribution in the activation of complement by mannan-coated ELISA plates, using inhibitory antibodies directed against C1q and MBL. It is likely that the relative contribution of C1q is strongly increased in donor serum containing high levels of IgG and IgM anti-mannan antibodies in combination with low levels of functional MBL. In such a situation, the contribution of C1q may mask the detection of deficiency of the LP unless CP activation is prevented. Therefore, inhibition of CP is crucial for a reliable functional LP assay.

Different strategies are conceivable for the inhibition of C1q-mediated complement activation in human serum. In the present study, we show C1q inhibition with mAb 2204, an anti-C1q monoclonal antibody that binds to the globular heads of C1q and blocks the interaction with immunoglobulins. Furthermore, Fab fragments from polyclonal rabbit anti-C1q antibodies, but not complete IgG, can be used to specifically inhibit CP activation. An alternative option is the use of C1q-inhibitory peptides (25). This option is under investigation in our laboratory.

Our studies indicate that incubation of low serum concentrations on immobilized mannan may also activate the alternative pathway. This may involve stabilization of spontaneously activated C3, in a complex with activated factor B and properdin, by its binding to mannan, in a similar way as is effected with other heavily glycosylated microbial products that activate the AP, such as LPS and zymosan. Activation of C3 in the absence of calcium, as well as the lack of detectable C4 activation, strongly suggest an MBL-independent mechanism. Additional studies indicated that binding of MBL in the presence of EGTA was undetectable (38). However, we can not totally exclude that small amounts of MBL binding under these conditions may trigger complement activation, which is subsequently strongly amplified by the alternative pathway at the level of C3. Since activation of the AP requires a high serum concentration, it is highly unlikely that similar MBL-independent complement activation is involved in the LP activity assay when serum is diluted at least 50-fold. This is clearly demonstrated in experiments showing that activation of C4, C3, and C5b-9 was completely undetectable in serum from an individual with a homozygous mutation
at codon 54 of the MBL-gene (BB genotype), although this serum had an intact AP activity.

Two members of the ficolin family, L-ficolin and H-ficolin (Hakata antigen) have been recently shown to interact with MASP proteins, and thereby activate complement via the lectin pathway (39; 40). Ficolins are multimeric proteins with a carbohydrate-binding fibrinogen-like domain. L-ficolin does not bind to mannan and is therefore not likely to be involved in complement activation induced by mannan. Both L-ficolin and H-ficolin are present in human serum. At present, there is no information available about the activity of ficolin-mediated complement activation in full serum. Development of such an assay is dependent on the identification of ficolin-specific ligands that are able to activate ficolin-MASP complexes.

The activity of the LP in human serum is determined by a number of variables, including the concentration and molecular structure of MBL and MASP proteins, the activity of complement proteins from C4 until C9, as well as the presence and activity of serum inhibitors of complement activation (41; 42). The assay we now describe enables the functional detection of important consequences of LP activation, i.e. opsonization of the target with complement activation, and formation of the membrane attack complex. Studies using recombinant MBL molecules clearly showed that structural mutations of the MBL gene lead to an impaired functional activity (14). In agreement with these data, we demonstrate that serum from donors with a mutation at codon 54 of the MBL gene (B genotype) has a defect in activation of the LP, which is in the homozygous mutant serum accompanied by a apparent failure of MBL to bind to the activating ligand mannan. Primarily in heterozygous individuals, the consequences of structural mutations may be highly variable, depending on the relative expression of the mutated and the wildtype gene. Therefore, functional assessment of LP activity most likely provides a more relevant marker for LP defects than analysis of mutations in the MBL gene and promoter region. Further studies are now underway to examine the relation between the different parameters involved in LP function and the resulting LP-mediated complement activation.

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REFERENCES


CHAPTER 2.2

Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency


ABSTRACT

Deficiency of mannose-binding lectin (MBL), a recognition molecule of the lectin pathway of complement, is associated with increased susceptibility to infections. The high frequency of MBL deficiency suggests that defective MBL-mediated innate immunity can be compensated by alternative defense strategies. To examine this hypothesis, complement activation by MBL-binding ligands was studied.

The results show that the prototypic MBL-ligand mannan can induce complement activation via both the lectin pathway and the classical pathway. Furthermore, antibody binding to mannan restored complement activation in MBL-deficient serum in a C1q-dependent manner. Cooperation between the classical pathway and the lectin pathway was also observed for complement activation by p60 from Listeria monocytogenes.

MBL pathway analysis at the levels of C4 and C5b-9 in the presence of classical pathway inhibition revealed a large variation of MBL pathway activity, depending on mbl2 gene polymorphisms. MBL pathway dysfunction in variant allele carriers is associated with reduced MBL-ligand binding and a relative increase of low molecular weight MBL.

These findings indicate that antibody-mediated classical pathway activation can compensate for impaired target opsonization via the MBL pathway in MBL-deficient individuals and imply that MBL deficiency may become clinically relevant in absence of a concomitant adaptive immune response.
INTRODUCTION

Recognition of pathogen-associated molecular patterns by molecules of innate immunity can lead to direct and early target elimination as well as to antigen presentation resulting into clearance via adaptive immunity. A number of pattern recognition molecules have been identified, such as lectin receptors, Toll-like receptors, and soluble opsonins including complement factors. The importance of the complement system in innate immune defense is clearly illustrated by a number of genetic complement deficiencies described both in humans and mice.

Activation of the complement cascade can take place via at least three pathways identified until now: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP). Whereas the LP and the AP primarily use a direct target recognition mechanism, the CP is mainly activated via binding of the initiating factor C1q to e.g. antigen-bound IgG or IgM antibodies. The LP can be initiated by mannose-binding lectin (MBL), which in a calcium-dependent way binds to carbohydrate ligands present on a large number of pathogens (reviewed in (1; 2)). Both MBL and C1q are composed of trimers that are assembled into larger structures. The collagenous domains of C1q and MBL bind to related serine proteases, being the serine proteases C1r and C1s for the C1 complex and the MBL-associated proteases MASP-1, MASP-2 and/or MASP-3 for the MBL complex (2; 3). Activation of both pathways leads to formation of the C3 convertase C4b2a. Recently, two members of the ficolin family, i.e. L-ficolin and H-ficolin, have also been shown to bind MASPs and to activate the LP of complement (2). In the present study, therefore, MBL-dependent activation of the LP is called the MBL pathway (MP).

Three MBL gene (mbl2) polymorphisms have been identified that are associated with MBL deficiency. These single nucleotide polymorphisms (SNP) are located in codon 54 (B genotype), codon 57 (C genotype), and codon 52 (D genotype) of the first exon, encoding the collagenous region of the MBL molecule (reviewed in (4; 5)). Experiments with recombinant MBL confirmed that these SNP affect the structure and function of MBL (6-8). Furthermore, SNP in the promoter and untranslated region of the mbl2 gene modify the basal serum level of MBL (9).

MBL deficiency has been identified as the basis of a common defect in opsonization of yeast-derived mannan (10), which is associated with decreased pathogen resistance (11; 12), particularly during early childhood (13). Among the complement deficiencies described in humans, deficiency of MBL has the highest frequency. Depending on ethnicity, the total allele frequency of the B, C and D allele may be above 40% (9). Since apparently these polymorphisms are not subject to a high negative selection pressure, it has been proposed that the polymorphisms are associated with host protection in certain situations (14). Furthermore, the clinical effect of MBL deficiency
is strongly dependent on the immune status of the individuals tested, and most MBL-deficient individuals are apparently healthy. Together, the data suggest that although MBL gene polymorphisms do have important functional consequences for activation of the MP of complement, most affected persons have alternative mechanisms for target recognition to reach a sufficient level of anti-microbial protection.

Recently, assays became available that allow detailed functional evaluation of the MP, thus allowing a thorough examination into the mechanisms involved in complement activation by the prototypic MBL-ligand mannan (15; 16). The present study shows that, although the function of the MP of complement activation can be strongly hampered by MBL gene polymorphisms, recognition of mannan by the complement system may still proceed in an MBL-independent but C1q-dependent manner. Similarly, we show that p60, a protein derived from *Listeria monocytogenes*, can activate the complement system via both MBL-dependent and MBL-independent mechanisms.

**RESULTS**

**Both C1q and MBL can support complement activation by mannan in human serum**

To characterize the mechanisms of complement activation by mannan in full human serum, serum samples from healthy donors who were genotyped for SNP of the *mbl2* gene were investigated for their capacity to activate C4 by mannan. C4 activation was observed in all sera examined, with a high inter-individual variability (Fig. 1A). Activation of C4 in A/B donors was significantly lower than in wildtype (A/A) donors (p < 0.01).

In order to assess complement activation by mannan via the MP only, activation of C4 by mannan was assessed in human serum in the presence of a C1q-blocking mAb, as recently described (16). The activation of autologous C4 via the MP was strongly dependent on the MBL genotype (Fig. 1B, p = 0.0002 in ANOVA), and was significantly hampered in A/B donors (p < 0.001) but not in A/D donors, as compared to A/A donors (Fig. 1B).

Initial strong activation of C4 by mannan could in a number of sera be inhibited to an undetectable level by inhibition of C1q (compare fig. 1A with 1B, for example donor A/C and donor B/D), indicating a clear contribution of C1q and the CP in complement activation by mannan. The relative contribution of C1q is significantly higher in sera with low activity of the MP than in sera with high activity of the MP (Fig. 1C, ANOVA p < 0.0001).
The activity of the CP, based on the activation of C4 induced by immobilized IgM (16), was high in all donor sera, with a low variation, and no difference between the sera of different MBL genotypes (ANOVA: $p = 0.87$) (Fig. 1D).

Since activation of C4 by mannan in a number of cases was C1q-dependent, the role of anti-mannan antibodies in complement activation by mannan was further studied. Serum levels of IgG and IgM anti-mannan antibodies are highly variable (16) and did not significantly differ between the MBL genotypes (not shown). Pre-incubation of mannan-coated plates with purified IgG (Fig. 2A) or IgM (Fig. 2B) induced a dose-dependent deposition of C4 on mannan upon addition of MBL-deficient serum. This activation of C4 was completely inhibited by a C1q-inhibitory mAb (Fig. 2), clearly indicating that mannan-binding IgG and IgM can restore complement activa-
C1q and MBL cooperate in complement activation by p60 of *Listeria monocytogenes*

Data presented above indicate that mannan, a major yeast antigen, can support activation of the complement system via both the CP and the MP. To extend these observations towards another microbial target, we examined activation of the complement system by protein p60 from *Listeria monocytogenes*. Purified human MBL showed a strong and dose-dependent binding to p60, which was completely inhibited by D-mannose but not L-mannose (Fig. 3A), indicating involvement of the lectin domain of MBL. Interestingly, also purified C1q showed a strong binding to immobilized p60 (Fig. 3B) but not to mannan (not shown). Complement activation was further studied using two groups of sera that were either sufficient or deficient for MP activity. C4 activation by listerial p60 was significantly higher in MBL-sufficient sera than in the MBL-deficient sera (Fig. 3C). In the presence of mAb 2204 for C1q inhibition, only sera with a functional MP activity showed activation of C4 (Fig. 3C). When C1q is inhibited, activation of C4 by p60 is completely blocked by D-mannose, strongly suggesting a complete dependence on MBL under these conditions (Fig. 3D). These data support an important role for both C1q and MBL in complement activation by *Listeria p60*.

Together, these results provide evidence for a contribution of the CP to complement activation by two different ligands for MBL, which can compensate for MBL dysfunction. In studies presented below the basis of MP dysfunction is further examined using MBL-specific assays that exclude participation of the CP.
Factors involved in the variability of MBL pathway activity in serum

Activation of the MP by mannan was subsequently assessed at its final stage. Formation of C5b-9 via the MP, as assessed in the presence of a C1q inhibitor, is strongly dependent on the MBL genotype (Fig. 4A). A significantly lower activity was observed in A/B donors (p < 0.001) but not in A/D donors, as compared to A/A donors. MP-mediated activation of C4 and activation of C5b-9 was strongly correlated (R = 0.89, P < 0.0001).

Next to MBL exon 1 polymorphisms, other factors considered to be involved in the extreme variation of MP activation by mannan in human serum were the MBL serum concentration, the capability of MBL to bind to mannan, the activity of the
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Figure 4. Functional characterization of the MBL pathway
MP activity assessed at the level of C5b-9 in the presence of mAb 2204 (A, dilutions starting from 1/100), MBL concentration (B), MBL binding to mannan (C, dilutions starting from 1/10), and MBL complex activity (D, dilutions starting from 1/50) in different sera. ANOVA: p < 0.001, A/A versus A/B p < 0.001

MBL-MASP complex, MBL promoter polymorphisms and MASP-2 polymorphisms. The MBL serum concentration (Fig. 4B), the capacity of MBL to bind to mannan (Fig. 4C) as well as the C4-cleaving activity of the MBL complex, as determined by exogenously added C4 (15) (Fig. 4D), were strongly decreased in carriers of MBL variant alleles. For all three parameters, A/B donors but not A/D donors showed a significant difference as compared to A/A donors.

Both in wildtype and variant serum, MP activity assessed at the level of C5b-9 correlated highly significantly with the MBL concentration (Fig. 5A), the MBL ligand binding activity (Fig. 5B), and the MBL complex activity (Fig. 5C), demonstrating that the availability of functionally active MBL is the major determinant of the activity of the MP in full human serum. Furthermore, MBL complex activity was strongly correlated to both the MBL concentration (Fig. 6A), and the capacity of MBL to bind to mannan (Fig. 6B), suggesting that impaired ligand binding is an important cause of low MBL complex activity in carriers of variant alleles.
Since our data indicate a functional MP defect in carriers of the A/B genotype, we further characterized the functional properties of MBL in serum from A/B donors. Serum samples from A/B donors were compared with serum samples from A/A donors having a comparable MBL concentration (Table 1). The MBL ligand binding capacity and the MBL complex activity of circulating MBL were significantly decreased in A/B donors as compared to A/A donors. This difference in MBL complex activity between wildtype and variant MBL is also illustrated in Fig. 6A.

MBL promoter polymorphisms have been identified that control the MBL serum concentration. Accordingly, A/A donors with the H/H promoter genotype show higher MBL levels than A/A donors with the L/L promoter genotype (Fig. 7A). Furthermore, MBL complex activity and MP activity were significantly higher in sera obtained from H/H donors than in sera obtained from L/L donors (Fig. 7A). These functional effects are presumably directly related to the effects of the promoter polymorphisms on MBL gene expression.

MBL promoter polymorphisms are in strong linkage disequilibrium with exon 1 polymorphisms. In this respect, the B genotype is always found in haplotypes car-

Figure 5. MBL pathway activity is dependent on the presence of functional MBL. MP activity is plotted against the MBL concentration (A; R = 0.74 (wildtype); R = 0.90 (variant)), MBL binding to mannan (B; R = 0.67 (wildtype); R = 0.90 (variant)) and MBL complex activity (C; R = 0.76 (wildtype); R = 0.91 (variant)), for sera obtained from MBL wildtype and variant individuals, as indicated. P < 0.0001 for all correlations.
rying the LYP allele, and the D genotype is found on the HYP haplotype (9). In donors with heterozygous exon 1 SNP, MBL promoter polymorphisms present in the wildtype allele will determine the relative expression of the wildtype and the variant allele. To directly assess the impact of the B and the D allele, sera from A/B donors and from A/D donors were compared with serum from A/A donors with the same promoter genotype, respectively. Sera from LYQ A/LYPB donors have a significantly lower MBL concentration and MP activity as compared to LYQ A/LYPA donors. Furthermore, sera from HYPA/HYPD donors have a significantly lower MBL concentration than sera from HYPA/HYPA donors (Fig. 7B).

Recently, a SNP in the MASP-2 gene was identified that can cause MASP-2 deficiency (17). Although the frequency of this variant allele was described to be 5.5%, all donors included in our study were homozygous carriers of the wildtype allele (assessed by PCR-RFLP, data not shown).

**Impaired MBL function is related to impaired MBL polymerization**

The molecular structure of MBL was examined in whole human serum from individuals with different genotypes by Western blotting (Fig. 8). Both wildtype and variant MBL showed a doublet between approximately 160 and 200 kDa, at variable amounts correlating with the MBL serum concentration. A number of high molecular weight bands (above ± 200 kDa) were observed only in those sera that show detectable MBL complex activity, also following prolonged exposure. In contrast, a double band was observed around 90 kDa that is predominantly present in carriers of variant alleles. Carriers of two variant alleles contained only low molecular weight MBL (up to ± 200 kDa), whereas a mixture of low and high molecular weight MBL was detected in heterozygous carriers of variant alleles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MBL genotype</th>
<th>Median (range)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBL concentration (ng/ml)</td>
<td>A/A</td>
<td>342 (99-607)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>287 (126-571)</td>
<td></td>
</tr>
<tr>
<td>MBL ligand binding capacity of circulating MBL (U/ng)</td>
<td>A/A</td>
<td>1.09 (0.34-2.49)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>0.25 (0.15-1.5)</td>
<td></td>
</tr>
<tr>
<td>MBL complex activity of circulating MBL (U/ng)</td>
<td>A/A</td>
<td>1.36 (0.99-1.98)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>0.83 (0.29-1.77)</td>
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A/B donors (n = 12) were compared to A/A donors (n = 11) matched on basis of similar MBL serum concentrations. The parameters given were calculated by dividing the MBL binding to mannan (U/ml) or the MBL complex activity (U/ml), by the MBL concentration (ng/ml), as indicated. Data were analyzed by using the Mann-Whitney U test.
Figure 6. MBL complex activity is determined by the availability of MBL binding to mannan. MBL complex activity is plotted against the MBL concentration (A; R = 0.88 (wildtype); R = 0.94 (variant)) and the level of MBL binding to mannan (B; R = 0.86 (wildtype); R = 0.96 (variant)) for sera obtained from MBL wildtype and variant individuals. P < 0.0001 for all correlations.

Figure 7. Functional effects of MBL promoter variants. Sera from A/A donors homozygous for the H or L promoter variant (A) and sera from A/B donors, A/D donors and A/A donors with identical promoter haplotype (B) are compared for different parameters of MBL function, as indicated.
The present study demonstrates that, although the MP of complement can be severely hampered by MBL deficiency, activation of the CP of complement via C1q and anti-carbohydrate antibodies can compensate for such a defect.

Epidemiological studies clearly indicated that MBL is an important factor of innate immune defense, both as a primary opsonin and as an activator of the complement cascade. In case of MBL deficiency, hampering the opsonization of targets with MBL and complement components, other molecules may support phagocytosis and presentation of carbohydrate antigens to the acquired immune system, thereby compensating for the lack of functional MBL. In this respect, recognition of carbohydrates on pathogens may also involve lectin receptors expressed on phagocytes, other soluble lectins, including collectins and ficolins (1; 18), and anti-carbohydrate antibodies.

Certain anti-carbohydrate antibodies are present at high levels in human serum, such as antibodies directed against the ABO blood group antigens and antibodies against the major xenoantigen, Galα1-3Gal. These anti-carbohydrate antibodies most likely originate from the continuous immune stimulation by bacteria of the gut flora. It is likely that similar mechanisms are responsible for the production of anti-mannan
antibodies in humans, in response to the high mannose structures commonly found on microbial surfaces. In the current study we show that anti-mannan antibodies are able to induce the CP of complement, thereby inducing complement activation in MBL-deficient serum until a level that is comparable to that of MBL-sufficient serum.

The presented data indicate that human sera display a high variability when tested for the activation of C4 by mannan, which is in agreement with findings of Super and Minchinton (19; 20). Our study indicates significant MBL-independent complement activation by mannan, which is dependent on C1q. In case of MBL dysfunction, C4 activation by mannan is dominated by activation of the CP, as is illustrated by an increasing relative contribution of C1q with decreasing MP activity. The contribution of the CP is most likely explained by the presence of anti-carbohydrate antibodies, which are present in human serum with a high inter-individual variation (16). Anti-carbohydrate antibodies may contribute to early host defense, and, when produced at a sufficient level, may compensate for the lack of functional MBL in the protection against at least part of the pathogens recognized by MBL. This could also explain why MBL-deficient young children, who do not yet produce sufficient levels of antibodies, are more prone to acquire infections than MBL-deficient adults (13).

In the present study we investigate the role of MBL and C1q in opsonization of mannan, a component of *Saccharomyces Cerevisiae*, as well as p60, a protein from *Listeria monocytogenes*. The latter microorganism is a facultative intracellular bacterium that can cause severe systemic infections, associated with e.g. septicemia and meningitis, in humans. Previous studies have shown binding of purified MBL (21) and C1q (22) to this microorganism, and C1q binding was shown to be involved in the uptake of *L. monocytogenes* by macrophages. We now show that the p60 protein of *L. monocytogenes* is able to activate the LP of complement via a direct interaction with MBL. Furthermore, C1q is also able to directly bind to this protein, activating the CP. Activation of the CP may be further promoted by antibodies against p60 which are present in human serum (23). In case of MBL deficiency, activation via the CP only results into a significantly lower level of opsonization. Interestingly, the p60 molecule has been implicated in host cell invasion, as well as in the phagocytosis of *L. monocytogenes* by dendritic cells (23). Our data indicate cooperation between the CP and the LP in complement activation by p60 from *L. monocytogenes*, thereby most likely promoting phagocytosis and bacterial killing. However, a role for complement and complement receptors in the invasion of this bacterium into non-phagocytic host cells can not be excluded.

The difference that we noticed between complement activation by mannan in the absence or in the presence of C1q inhibition confirms previous suggestions that the CP is likely to be involved in complement activation assays using mannan.
Therefore, assays have been developed, which are used in the present study, in which the CP was inhibited, either by the use of high ionic strength buffer (15), or by including a blocking mAb directed against C1q (16). Previous studies have assessed complement activation by mannan without excluding interference of the CP (19; 20). Minchinton et al. recently presented a detailed study concerning the capacity of serum from healthy donors with different MBL genotypes to activate C4 by mannan, using incubation of serum samples at physiologic ionic strength, followed by a second incubation with an MBL-deficient complement source (20). In agreement with our study, this method also revealed that C4 activating capacity was severely hampered in carriers of MBL variant alleles.

In the present study we evaluate specific MP activation of the whole complement cascade, up to C5b-9 formation, using autologous complement components and an inhibitor of C1q. Our results indicate that heterozygous and homozygous expression of the B allele is associated with low MBL serum concentrations, low MBL binding to mannan and low MBL complex activity, resulting into hampered activation of C4 and C5b-9 via the MP. These findings raise the question of the primary cause of impaired MBL function in individuals with structural MBL polymorphisms.

The serum level of MBL is a strong determinant of both MBL complex activity (15) and MP activity. In our study, decreased serum concentrations of MBL were observed in donors with two structural mutations as well as in both A/D and A/B donors, when compared to wildtype donors with the same promoter haplotype.

MBL in serum shows a wide range of molecular weights, and MBL gene mutations are associated with low molecular weight MBL (24). We show that low molecular weight MBL, which may represent MBL oligomers consisting of up to two trimers (8), is present in variant serum and virtually absent in wildtype serum. Furthermore, the presence of MBL with a molecular weight above 200 kDa, presumably representing molecules consisting of at least three trimers, is required for MBL complex activity and activation of the MP of complement in serum, and these bands are lacking in serum from carriers of two variant alleles. These results agree with recently obtained data with human recombinant MBL (8). Furthermore, the MASP-2 content and the ability to cleave C4 were reported to be highest in human MBL complexes of about 345 kDa (3).

Experiments with recombinant human and rat MBL also point to functional defects of the variant molecules, showing impaired complement activation and MASP binding (6-8). Our data show that MBL binding to mannan and MBL-MASP complex activity of circulating MBL are strongly decreased in carriers of MBL variant alleles. This difference is apparent in A/B donors and in donors with a double MBL mutation (B/B and B/D) but not in A/D donors, which is in agreement with data from Minchinton et al. (20), and which was also suggested by data obtained with recombinant rat MBL.
Molecular and immunological characteristics of MBL

(7). Taken together, the intrinsic defects that we detect in circulating variant MBL are a relative increase in low molecular weight MBL and an impaired ability to bind to mannann, resulting into reduced MBL complex activity and hampered activation of C4 and C5b-9. Impaired ligand binding most likely results from a lower avidity that is inherent to smaller MBL molecules with less ligand-binding domains. Low molecular weight MBL in carriers of variant alleles, in association with impaired ligand binding, was also recently shown by gel filtration (24; 25).

Associations between MBL deficiency and increased susceptibility to infectious diseases have been frequently reported, in otherwise healthy subjects (10-12) and, more strongly, in patients with additional immunological defects or chronic diseases (26-28). In these studies, MBL deficiency was always defined on basis of the MBL genotype, the MBL concentration, or both, and genetic associations could be observed both for homozygous and heterozygous carriers of MBL variant alleles (10; 11; 28).

The present study shows that, also when the MBL haplotype is taken into account, MP activity shows a much larger variation than CP activity. Sera from healthy donors with an identical MBL haplotype can have a 10-fold difference in MP activity (Fig. 7 and unpublished results). Additional polymorphisms in the genes of MBL and/or MBL-associated molecules could play a role in this variation. The recently characterized variant allele of MASP-2 (17) was not present in the donors examined in our study, suggesting that this variant may be more prevalent in the Scandinavian population.

In conclusion, activation of the complement cascade via the MP is critically dependent on the availability of MBL that is able to bind to its ligands. However, MP dysfunction is not necessarily associated with inadequate opsonization, since anti-carbohydrate antibodies and the CP of complement can take over this function. Antibodies, C1q and MBL can cooperate in early host defense by simultaneous activation of parallel pathways of the complement system. Therefore, MBL deficiency primarily may become clinically relevant in situations without a concomitant adaptive immune response.

MATERIALS AND METHODS

Human materials

Blood was taken from 70 healthy donors. Serum was immediately aliquotted and frozen at -80°C. Heparinized blood was used for genomic DNA isolation (16).
MBL genotyping

MBL exon 1 SNP were identified using an oligonucleotide ligation assay (16) and confirmed by PCR using sequence-specific priming (29). Furthermore, the regulatory genetic variants H/L (-550), X/Y (-221), and P/Q (+4) (9) were typed as described (29).

Functional assessment of complement activation

Functional activity of the MP and the CP were assessed using methods that were previously described in detail (16), using plates coated with mannan and human IgM to assess MP and CP activity, respectively. MP function was assessed in the presence of a blocking mAb directed against C1q (mAb 2204, 20 µg/ml; kindly provided by Dr. C.E. Hack, Sanquin Research, Amsterdam). In some experiments, complement activation on mannan was assessed in the absence of mAb 2204, as indicated. Complement activation was detected using monoclonal antibodies directed against C4 and C5b-9, as described (16). For specific experiments, mannan-coated plates were pre-incubated with IgG and IgM purified from human donor plasma, diluted in PBS containing 1% BSA, 0.05% Tween 20, and 10 mM EDTA.

Binding of MBL to mannan was assessed following incubation with human serum diluted in a Calcium-containing buffer (GVB++, using dig-conjugated mAb 3E7 (anti-human MBL) as described (16).

Complement activation by p60 from Listeria monocytogenes

ELISA plates were coated with p60 (5 µg/ml), a protein from Listeria monocytogenes expressed as described before ((30), kindly provided by Dr. S.H.E. Kaufmann, Berlin, Germany) in coating buffer (100 mM NaCO₃/ NaHCO₃, pH 9.6). Binding sites were saturated with 1% BSA in PBS. Binding of MBL (diluted in GVB++) and C1q (diluted in PBS/ 1% BSA / 0.05% Tween 20) (both purified from normal donor plasma), was assessed using mAb 3E7 and mAb 2214 (anti-human C1q, from Dr. C.E. Hack), respectively, both conjugated to dig. Deposition of C4 in the presence or absence of mAb 2204, D-mannose or L-mannose (both used at 100 mM, from Sigma) was assessed.

MBL concentration

The concentration of MBL in serum was assessed by sandwich ELISA. Plates were coated with 3E7 (mAb anti-MBL) at 5 µg/ml. Sera were diluted in PBS containing 0.05% Tween-20 and 1% BSA. MBL was detected using rabbit IgG anti-MBL (20 µg/ml, prepared by immunization of rabbits with purified human MBL), followed by HRP-conjugated goat anti-rabbit IgG (from Jackson ImmunoResearch Laboratories, West Grove, PA, USA).
Molecular and immunological characteristics of MBL

MBL complex activity

MBL complex activity was assessed using the method described by Petersen et al. (15) with slight modifications. Mannan-coated plates were incubated with serum, diluted in GVB++ containing 1 M of NaCl, during 16 hours at 4°C. Plates were washed with PBS/Tween containing 5 mM CaCl₂, followed by incubation with purified C4 (1 µg/ml), diluted in GVB++, for 1 hour at 37°C. Activation of C4 was assessed.

Western blotting

Human serum (1.5 µl) was subjected to SDS-PAGE using a 4 to 15% polyacrylamide gradient gel (Criterion Pre-cast gel, Tris-HCl, from Bio-Rad Laboratories, Richmond, CA, USA) under non-reducing conditions. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Bedford, USA) using a semi-dry blotting procedure. Membranes were blocked with PBS/0.05% Tween 20/2% Casein followed by incubation with mAb 3E7 (1 µg/ml) for 16 hours at 4°C and HRP-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) for 2 hours at room temperature. Development of blots was performed with Supersignal (Pierce Chemical Co., Rockford, IL, USA) and exposure to Hyperfilms (Amersham Pharmacia Biotech).

Calculations and statistical analysis

All sera were analyzed in at least two dilutions in duplicates. Functional activity was expressed in units per ml, based on serial dilutions of a human pool serum used as a standard (set at 1000 U/ml). The activity was calculated using parameters of linear regression following log-transformation of arbitrary units and logit-transformation of OD values. Statistical analysis was performed with GraphPad Prism 3.03 using non-parametric tests. Differences were evaluated by the Mann Whitney U test or by analysis of variance (ANOVA), using the Kruskal Wallis test and Dunn’s correction for multiple comparisons. Correlation was evaluated using the Spearman Rank correlation coefficient ($R$). Results were considered as statistically significant when $p$ values were below 0.05.

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CHAPTER 2.3

Human immunoglobulin A activates the complement system via the mannan-binding lectin pathway

Anja Roos, Lee H. Bouwman, Danielle J. van Gijswijk-Janssen, Maria C. Faber-Krol, Gregory L. Stahl, Mohamed R. Daha

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ABSTRACT

The recently identified lectin pathway of the complement system, initiated by binding of mannan-binding lectin (MBL) to its ligands, is a key component of innate immunity. MBL-deficient individuals show an increased susceptibility for infections, especially of the mucosal system. We examined whether IgA, an important mediator of mucosal immunity, activates the complement system via the lectin pathway. Our results indicate a dose-dependent binding of MBL to polymeric but not monomeric IgA coated in microtiter plates. This interaction involves the carbohydrate recognition domain of MBL, since it was calcium-dependent and inhibited by mannose and by mAb against this domain of MBL. Binding of MBL to IgA induces complement activation, as demonstrated by a dose-dependent deposition of C4 and C3 upon addition of a complement source. The MBL concentrations required for IgA-induced C4 and C3 activation are well below the normal MBL plasma concentrations. In line with these experiments, serum from individuals having mutations in the MBL-gene showed significantly less activation of C4 by IgA and mannan than serum from “wildtype” individuals. We conclude that MBL binding to IgA results in complement activation, which is proposed to lead to a synergistic action of MBL and IgA in antimicrobial defense. Furthermore, our results may explain glomerular complement deposition in IgA nephropathy.
INTRODUCTION

The complement system is an important component of host defense. Activation of the complement cascade takes place upon the interaction of complement components with a variety of pathogens, either directly or via antibodies bound to pathogen antigens. Three different pathways for complement activation have been described, the classical pathway, the alternative pathway and the lectin pathway, of which the latter is most recently identified. The lectin pathway is mainly driven by mannan-binding lectin (MBL).

MBL, a member of the collectin family (1), is a C-type lectin present in serum as a part of a large pro-enzymatic complex. The MBL protein consists of three to six identical homotrimeric subunits. Each trimer is composed of a collagen-like tail part and a globular head part containing a carbohydrate recognition domain (CRD). The collagen-like part of MBL interacts with the MBL-associated serine proteases MASP-1 (2), MASP-2 (3), and MASP-3 (4). These enzymes are responsible for the complement-activating properties of the MBL complex, by the cleavage of C4, C2 and C3 (5). The CRD of MBL is able to bind in a calcium-dependent way to a number of saccharides, such as D-mannose, L-fucose, and N-acetyl-glucosamine (1). MBL binding to a ligand induces activation of the MASP enzymes, leading to complement activation up to the terminal pathway. Both the structural and functional properties of MBL are strikingly similar to those of C1q, the recognition unit of the classical complement pathway.

Genetic mutations in the MBL gene are present with a high frequency in the human population (6). Until now, three different point mutations have been described which lead to the production of MBL with structural aberrations and impaired complement-activating properties (6; 7). Heterozygous and homozygous expression of these mutant alleles is associated with an enhanced incidence of a range of infections, in both children and adults (8; 9). In this spectrum of diseases, mucosal infections, occurring in the respiratory tract and the gastrointestinal tract, are common. Furthermore, mutations in the MBL gene have a significant negative impact on chronic diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and cystic fibrosis, resulting in an increased incidence of complicating infections and/or a worse outcome (10-12). These studies indicate the importance of the lectin pathway of complement activation in anti-microbial defense. In line of this function of the lectin pathway, MBL is able to bind directly to a number of microorganisms, via the carbohydrates expressed on their surface (1; 13). Upon binding, complement activation takes place, leading to either direct elimination via the terminal complement pathway, or opsonization and phagocytosis.
In the mucosal immune system, a major factor of defense is immunoglobulin A. It is present in plasma at a concentration of about 2 mg/ml, and it is secreted at mucosal surfaces throughout the body where it is postulated to play an important role as a defense mechanism against invading microorganisms (14; 15). Upon interaction of IgA with pathogens, the IgA molecule can have diverse effector functions, including the direct prevention of invasion of microorganisms, the interaction with the phagocytic IgA Fc receptor CD89, and complement activation.

Complement activation by IgA has been previously shown to involve the alternative but not the classical complement pathway (16; 17). No data are available concerning the possible involvement of the lectin pathway. A classical disease involving IgA and complement activation is primary IgA nephropathy, a common renal disease involving mesangial deposition of IgA and complement components, resulting in end stage renal failure in about 30% of the patients (18). Recent studies indicate the presence of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19; 20).

Therefore, we examined whether IgA induces complement activation via a possible interaction with MBL. Our results demonstrate activation of the lectin pathway by IgA. This novel interaction in the immune system is proposed to contribute to the roles of MBL and IgA in host defense, as well as to the pathogenesis of IgA nephropathy.

**MATERIALS AND METHODS**

**Purification of MBL/MAFP complexes**

MBL and its associated proteases were purified from pooled plasma obtained from healthy human donors, essentially as described by Tan et al. (21). In brief, a precipitation step was performed using polyethylene glycol 3350 (Sigma, St. Louis, MO; 7% (w/v)). The precipitate was dissolved in TBS-T/Ca\(^{2+}\) (50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, 10 mM CaCl\(_2\), pH 7.8) and incubated for 18 hours at 4°C with mannan-agarose (Sigma; equilibrated with TBS-T/Ca\(^{2+}\)). After extensive washing with TBS-T/Ca\(^{2+}\)/1M NaCl, bound proteins were eluted using TBS-T containing 10 mM EDTA. Fractions containing MBL, as determined by ELISA, were pooled and concentrated. To remove contaminating immunoglobulins, the MBL preparation was absorbed using a mixed absorbent consisting of 4E8 (mAb anti-IgA, produced in the laboratory of Nephrology, Leiden, the Netherlands) coupled to Biogel A5 (Biorad, Richmond, CA, USA), HB57 (mAb anti-IgM, hybridoma obtained from the American Type Culture Collection (ATCC)) coupled to Biogel A5, and protein G coupled to Sepharose (from Pharmacia, Uppsala, Sweden). The resulting MBL preparation con-
tained negligible amounts of IgA (< 0.5%) whereas IgG and IgM were undetectable. Furthermore, the MBL preparation did not contain any detectable C1q, as determined by single radial immunodiffusion and by a sensitive C1q-specific hemolytic assay. This purification method results in co-purification of MASP proteins, as shown by Western blotting using rabbit anti-MASP-1 antibodies, prepared as described (3), and rabbit anti-MASP-2 antibodies (kindly provided by Dr. R. Sim, Oxford, UK) as well as by a C4 consumption assays (not shown). The resulting MBL-MASP preparation was subjected to ELISA to determine the MBL concentration (as described below) and subsequently used in all experiments.

Purification of human IgA

IgA was purified from pooled normal human serum (NHS) or recalcified donor plasma as described by Hiemstra et al. (16) with minor modifications. In brief, the majority of serum proteins were removed by dialysis against H2O, and precipitation by ZnSO4. Proteins in the supernatant were precipitated using glycine and (NH4)2SO4, dialyzed against TE buffer (10 mM Tris, 2 mM EDTA, pH 7.8) and loaded on a DEAE-Sephacel column (Pharmacia). IgA was eluted with a linear salt gradient (conductivity 1-20 mS). IgA-containing fractions, as determined by ELISA, were pooled, concentrated and further purified by gel filtration, using a Sephacryl S-300 column (Pharmacia). Veronal-buffered saline (VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 2 mM EDTA was used as a running buffer. Fractions were tested for IgA by ELISA; IgA of different molecular sizes, i.e. monomeric, dimeric, and polymeric IgA were pooled separately on basis of its position in the elution profile. Reanalysis of these different IgA preparations indicated that this molecular size is stable: typically > 90% runs at the same position in the elution profile. Contamination with IgG and IgM was below 0.4% on a weight basis, as determined by ELISA. Experiments were performed with polymeric IgA unless otherwise indicated.

Purification of human IgG and IgM

Human IgG was purified from outdated plasma obtained from healthy donors as described previously (22). Human IgM was purified from human serum by euglobulin precipitation and anion exchange chromatography, as described (22), followed by cation exchange chromatography, using CM-Sephadex C-50 (Pharmacia), and gel filtration, using a Sephacryl S-300 column (Pharmacia). The IgG preparation was free of any detectable IgA and IgM, and the IgM preparation was free of any detectable IgA and IgG.
Purification of functionally active C4

Freshly obtained NHS (120 ml) was adjusted with TEB-buffer (10 mM Tris, 2 mM EDTA, 1 mM benzamidine hydrochloride hydrate (Sigma)) to pH 7.8 and conductivity of 4.0 mS. This sample was loaded on a DEAE-sephacel column (5 x 10 cm) which was previously equilibrated with TEB buffer at pH 7.8 and mS 4.0. The column was extensively washed with the same buffer, followed by elution with a linear salt gradient with a conductivity from 4.0 mS to 25 mS. Fractions were tested for the presence of C4 using a hemolytic assay. In this assay, serum from C4-deficient guinea pig is used as a complement source and sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC antibodies as targets. Lytic activity of an excess amount of C4-deficient serum can be restored by addition of a limiting amount of C4. Peak fractions containing C4 (at 15 mS) were pooled and concentrated. The concentration of C4 was determined by single radial immunodiffusion.

Human serum containing wildtype or mutated MBL

Serum samples were obtained from 5 healthy donors having the wildtype genotype at codon 52, 54, and 57 from the first exon of the MBL gene (A-genotype) as well as from 6 healthy donors with a homozygous (n = 2) or heterozygous (n = 4) mutation at codon 54 (B-genotype) (6). These genotypes were identified by an oligonucleotide ligation assay (A. Roos et al., manuscript in preparation) and were confirmed by DNA sequencing.

ELISA protocol

For all ELISA assays, Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated using coatingbuffer (100 mM Na₂CO₃/ NaHCO₃, pH 9.6), for 16 hours at room temperature or for 2 hours at 37°C. After each step, plates were washed three times with PBS containing 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS containing 1% BSA. Unless otherwise indicated, all subsequent steps were incubated in PBS containing 0.05% Tween 20 and 1% BSA, for one hour at 37°C. Detection antibodies were conjugated to digoxigenin (dig) using digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of binding of antibodies conjugated to dig was performed by HRP-conjugated rabbit anti-dig antibodies (Fab fragments, from Boehringer Mannheim). Enzyme activity of HRP was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).
Molecular and immunological characteristics of MBL

MBL detection ELISA

Plates were coated with 3E7 (mAb anti-MBL (mouse IgG1, kindly provided by Dr. T. Fujita, Fukushima, Japan) at 5 µg/ml. Samples containing MBL were incubated, followed by detection with dig-conjugated 3E7. A calibration line was produced using pooled human serum from healthy donors with a known concentration of MBL (kindly provided by Dr. P. Garred, Copenhagen, Denmark).

MBL binding to IgA or mannan

IgA was coated at 5 µg/ml, unless otherwise indicated. Mannan (from Saccharomyces Cerevisiae; Sigma (M7504)) was coated at 100 µg/ml. As a negative control, BSA (Sigma) was coated at 10 µg/ml. After blocking with BSA, MBL was incubated in BVB++ (VBS, 0.5 mM MgCl$_2$, 1 mM CaCl$_2$, 0.05% Tween-20, 1% BSA, pH 7.5) for one hour at 37°C. MBL binding was detected using dig-conjugated 3E7. In some experiments, MBL was pre-incubated (30 minutes, 20°C) in a calcium-free buffer (BVB/MgEGTA: VBS, 5 mM MgCl$_2$, 0.05% Tween-20, 1% BSA, 10 mM EGTA) or in BVB++ containing D-mannose, L-fucose, N-acetyl-glucosamine, or N-acetyl-galactosamine (from Sigma), followed by addition of the mixture to the plates. Saccharides were applied at a concentration of 100 mM unless otherwise indicated. Additional inhibition studies were performed using purified mouse mAb directed against MBL (1C10 and 3F8 (23)), which were incubated together with MBL at concentrations between 2 and 20 µg/ml.

Analysis of complement activation by MBL

Activation of complement via MBL was assessed as follows. Plates were coated with IgA, mannan, or BSA, blocked by BSA and, in some cases, incubated with MBL as described above. Subsequently, plates were incubated with 2% NHS as a complement source, diluted in BVB/MgEGTA, for one hour at 37°C. Deposition of C3 was detected by dig-conjugated RFK22 (mAb anti-human C3 (mouse IgG1) produced at the laboratory of Nephrology, Leiden, the Netherlands). Alternatively, C4 activation was assessed using a method adapted from Vorup-Jensen et al. (24). For these experiments, MBL, diluted in BVB++, was incubated for one hour at 37°C and for 16 hours at 4°C. Plates were washed with PBS / 5 mM CaCl$_2$ / 0.05% Tween-20, and C4 was added (1 µg/ml, diluted in BVB++ containing 1 mM MgCl$_2$ and 2 mM CaCl$_2$). C4 binding was detected with affinity-purified goat anti-human C4 antibodies conjugated to dig, or with dig-conjugated C4-4A (mAb anti-C4, kindly provided by Dr. C.E. Hack, Amsterdam, the Netherlands). In some experiments, activation of C4 was assessed directly in human serum. For this assay, all washing and incubation steps were performed in the absence of Tween 20, which reduced non-specific staining. Plates were coated with IgA, mannan, IgG (5 µg/ml) or IgM (5 µg/ml), washed
with PBS, and blocked by 1% gelatin in PBS. Serum was diluted in VBS containing 2 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 0.1% gelatin and incubated for 1 hour at 37°C. Subsequently, C4 binding was detected, using PBS / 1% BSA as a dilution buffer for antibody conjugates.

**Statistical analysis**

Differences in C4 activation between sera from two groups of donors (i.e. either MBL-wildtype or MBL-mutant genotype) were analyzed using a t test and are considered statistically significant when p values are less than 0.05.

**RESULTS**

**Interaction of MBL with IgA**

The binding of MBL to IgA was studied using microtiterplates coated with purified human IgA. Addition of MBL resulted in a dose-dependent binding to IgA, but not to a control coating with BSA only (Fig. 1A). Binding was clearly detectable at an MBL concentration of 20 ng/ml. Coating of different concentrations of IgA followed by incubation with a fixed concentration of MBL revealed that MBL binding was maximal at an IgA coating concentration of 5 µg/ml (Fig. 1B).

The characteristics of the interaction between MBL and IgA were studied by pre-incubating MBL in the presence of various inhibitors. Pre-incubation with D-mannose, L-fucose, or N-acetyl-glucosamine (GlcNAc), but not N-acetyl-galactosamine (GalNAc) blocked the binding of MBL to IgA and to its major ligand mannan (Fig. 1C). This inhibition by saccharides was dose-dependent: IC50 values for mannose and GlcNAc were between 5 and 10 mM for binding of MBL to IgA (Fig. 1D) and to mannan (not shown). Furthermore, incubation of MBL in a calcium-free buffer containing MgEGTA prevented binding of MBL to IgA and to mannan (Fig. 1C). Binding of MBL to IgA is calcium-dependent and reaches a plateau at 1 mM CaCl$_2$ (Fig. 1E). This concentration was chosen for further assays. These results indicate a calcium-dependent interaction of the CRD of MBL with human IgA.

**IgA activates the complement system via the lectin pathway**

In order to assess whether the interaction of MBL with IgA induces complement activation, activation of C4 and C3 was studied by ELISA. For experiments studying activation of C3, NHS diluted in a MgEGTA-containing buffer was used as a complement source. The use of MgEGTA prevented activation of the classical pathway and the lectin pathway in the complement source that may occur irrespective of the MBL that was previously bound to the coating, resulting in low background levels.
Figure 1. Binding of MBL to immobilized IgA. Microtiterplates were coated with either IgA or BSA, as indicated. MBL binding was detected by ELISA. **A**, MBL was added to coated IgA (5 µg/ml) or BSA at concentrations as indicated. Data represent mean ± SD from 4 independent experiments. **B**, IgA was coated at various concentrations, and binding of a fixed concentration of MBL (2 µg/ml) was detected. Data represent mean ± SD from two independent experiments. **C**, Wells were coated with mannan or IgA, and incubated with MBL (0.1 µg/ml on mannan; 1 µg/ml on IgA) either under standard assay conditions (control) or in the presence of MgEGTA, D-mannose, L-fucose, N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc) as indicated in Materials and Methods. The percentage inhibition of MBL binding to IgA or mannan was calculated using the following formula: 100 – (100 * (MBL binding (+ inhibitor) – MBL binding to BSA)) / (MBL binding (control) - MBL binding to BSA)), using OD values measured at 415 nm. None of the inhibitors affected the background binding of MBL to BSA. Mean and SD are shown of triplicate wells of a representative experiment. Similar results were obtained in 5 independent experiments. **D**, Similar experiment as shown in C, but the inhibitors were applied at different concentrations, as indicated (mean ± SD from two independent experiments). **E**, Wells were incubated with MBL (1 µg/ml) in the presence or absence of different concentrations of CaCl₂ as indicated. Data represent mean ± SD from two out of three similar experiments.
Figure 2. Binding of MBL to IgA induces complement activation. Wells were coated with IgA, mannan or BSA and in the first step incubated with MBL-MASP complexes in various concentrations, as indicated, followed by addition of a complement source in the second step. For A-D, the second step consisted of NHS (2% in VBS/BSA/Tween/MgEGTA), which was followed by detection of binding of MBL (A, B) or deposition of C3 (C, D). Alternatively, purified C4 was added in the second step, followed by detection of C4 binding (E, F). Please note that MBL concentrations on the X-axis in E and F are in nanogram per ml. The results are representative for at least three independent experiments.
Binding of MBL to IgA and to mannan, which was first achieved in the presence of calcium, was clearly detectable after a one-hour incubation with serum in the presence of MgEGTA (Fig. 2A, B), as has been previously reported for the binding of MBL to mannan-coated erythrocytes (25). Using these conditions, binding of purified MBL-MASP complexes induced a concentration-dependent deposition of C3 on coated IgA (Fig. 2C) and on coated mannan (Fig. 2D) upon addition of serum. Coated mannan requires about 10 times less MBL than coated IgA to induce the same level of MBL binding and C3 deposition (Fig. 2A, C versus 2B, D).

MBL binding to IgA and mannan also resulted in activation of C4. After binding of MBL-MASP complexes to either IgA (Fig. 2E) or mannan (Fig. 2F), addition of purified C4 resulted in a dose-dependent deposition of C4 on the coating. C4 activation

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

Figure 3. Complement activation is dependent on MBL binding. Plates were coated with mannan or IgA, followed by incubation with MBL in the presence of inhibitors as described at figure 1C. A, MBL was incubated at 1 µg/ml and 0.1 µg/ml on IgA and mannan, respectively, followed by addition of NHS (2% in VBS/BSA/Tween/MgEGTA) and assessment of C3 deposition. B, MBL was incubated at 2 ng/ml and 0.1 ng/ml on IgA and mannan, respectively, followed by addition of C4 and assessment of C4 binding. Results in A and B are mean ± SD of one out of two or three triplicate experiments. C, Similar experiment as shown in fig. 3B, but the saccharides were applied at different concentrations, as indicated. D, MBL was incubated on IgA and mannan in the presence or absence of mAb anti-MBL (3F8 and 1C10), as indicated. Binding of MBL and activation of C3 and C4 was assessed as described at fig. 1 and at fig. 3A and B, respectively. Anti-MBL antibodies were used at 20 µg/ml (MBL binding experiments) or at 2 µg/ml (complement activation experiments). Results represent the mean ± SD of triplicate experiments. Inhibition was calculated as described at fig. 1C.
was detectable at MBL concentrations between 0.01 and 1 ng/ml, depending on the coating used. MASP enzymes required for activation of C4 are present in the MBL preparation as demonstrated by its direct ability to induce C4 consumption in the fluid phase (not shown).

The results presented above strongly suggest that activation of C3 and C4 is induced by the binding of MBL and associated MASPs to IgA or mannan. To further establish this, the MBL preparation was pre-incubated on coated IgA or mannan in the presence of inhibitors, followed by addition of a complement source and analysis of deposition of C3 (Fig. 3A) and C4 (Fig. 3B, C). As expected for an MBL-dependent mechanism, pre-incubation of MBL with mannose, fucose and GlcNAc, but not GalNAc, blocked the activation of C3 and C4, both on IgA and mannan. Inhibition of C4 activation by mannose and GlcNAc was dose-dependent, and 50% inhibition was reached at saccharide concentrations between 5 and 10 mM, both on IgA (Fig. 3C) and on mannan (not shown). Similar dose-response relationships were observed for saccharide inhibition of C3 activation (not shown).

Incubation of MBL on mannan performed in the presence of MgEGTA completely inhibited subsequent C3 and C4 activation (Fig. 3A, B). On IgA, similar results were obtained for C4 deposition (Fig. 3B), whereas deposition of C3 was inhibited for a major part, but not completely (Fig. 3A).

To further prove the MBL-dependence of C3 and C4 activation on IgA and mannan, additional blocking studies were performed using mAb anti-MBL. Two different MBL-specific mAb were used (Fig. 3D): 3F8 that blocks MBL-mediated complement activation and, as a control, 1C10 that binds to MBL but does not block its function.
(23). The mAb 3F8 totally inhibited the binding of MBL as well as the activation of C3 and C4 on IgA and on mannan, whereas mAb 1C10 did not have any effect.

Complement activation is known to be predominantly a function of polymeric IgA (26). We tested the different molecular sizes of IgA for their ability to activate the lectin pathway. Polymeric IgA is superior to dimeric IgA in activation of the lectin pathway (Fig. 4). No significant activation could be detected by monomeric IgA.

Previous studies have shown that IgA can activate the alternative pathway in serum in the presence of MgEGTA (16). Only the alternative pathway, but not the classical pathway nor the lectin pathway, can proceed in the absence of Ca\(^{2+}\). To examine the combined contribution of the lectin pathway and the alternative pathway to activation of C3 by IgA, complement activation was studied with or without a pre-incubation with MBL. Serum incubated in the presence of MgEGTA, but in the absence of MBL, induced a clear deposition of C3 on IgA (Fig. 5), in agreement with previously published data (27). Pre-incubation with MBL in a calcium-containing buffer enhanced the deposition of C3 dose-dependently. Deposition of C3 was reduced to background levels when EDTA was present in the complement source (not shown).

![Figure 5](image-url)

Figure 5. IgA activates both the lectin pathway and the alternative pathway. Plates were coated with IgA or BSA, as indicated, and incubated in the presence or absence of MBL, followed by NHS in different concentrations in VBS/BSA/Tween/MgEGTA. C3 deposition was detected. Similar results were obtained in two experiments.
Serum containing mutated MBL has a partial defect in the activation of C4 by mannan and by IgA

MBL derived from individuals with mutations in exon 1 of the MBL gene has an impaired ability to activate the complement system (7). In order to examine whether this defect also hampers complement activation by IgA, serum from MBL wildtype donors (n = 5) was compared with that from donors with a mutant genotype (homozygous (n = 2; squares)) or heterozygous (n = 4) point mutations at codon 54 in a C4 activation assay. In comparison to the control group, serum from donors having MBL gene mutations induced significantly less C4 activation both on IgA (p = 0.015) and on mannan (p = 0.001) (Fig. 6). However, when plates were coated with human IgG of IgM, as activators of the classical complement pathway, both groups of sera induced a similar level of C4 activation.

**DISCUSSION**

The present study demonstrates that the CRD of MBL can bind to IgA and thereby activate the complement system via the lectin pathway. We propose that this interaction between the lectin pathway and IgA may function as a novel link between the innate and the adaptive immune system. Furthermore, the interaction between
MBL and IgA is expected to contribute to mesangial complement deposition in IgA nephropathy.

Our results demonstrate a calcium-dependent interaction of the CRD of MBL with IgA. This binding was evident at concentrations well below the mean MBL plasma concentration in healthy individuals, which is approximately 1.5 µg/ml. The carbohydrate specificity of the MBL-IgA interaction is similar to that of the interaction of MBL with mannan, and is consistent with the known specificity of MBL (1). Binding of MBL to IgA induced lectin pathway activation, as demonstrated by activation of C4 and C3. Complement activation on mannan and IgA-coated plates was inhibited by incubating the MBL preparation in the presence of mannose, in the absence of calcium, or in the presence of an MBL-blocking mAb, which is fully consistent with an MBL-dependent mechanism.

Although it has been demonstrated that MASP-1 can directly activate C3 in the fluid phase (5), we could not detect deposition of activated C3 when mannan-coated plates were incubated consecutively with MBL/MASP complexes and purified C3 (not shown), in agreement with data reported by Vorup-Jensen et al. (24). This discrepancy is most likely due to differences in the experimental settings, including the method of detection of C3 activation and the concentration and activity of MASP-1 present. In contrast, activation of C4 was readily detectable after incubation with purified C4 under similar conditions, both on IgA and on mannan. Activation of C3 was demonstrated when NHS was used as a complement source. In the latter experiment, we show that MBL binding to plate-coated IgA or mannan is preserved during a one-hour incubation step with a calcium-free buffer, although calcium is required to establish the primary interaction of MBL with its ligands. Similar characteristics are known for the binding of MBL to mannan-coated erythrocytes (25). The MBL-MASP interaction, which is required for complement activation, is also stable in a calcium-free environment (24).

C3 activation by IgA was also demonstrated when serum was used as a complement source in the absence of calcium and without pre-incubation with MBL. Calcium-independent C3 activation is consistent with activation of the alternative pathway by IgA, which is in agreement with previously published data (16, 27). Apparently, different complement activation pathways cooperate to induce activation of C3 by IgA.

To further establish a role for the lectin pathway in complement activation by IgA in whole serum, we compared sera obtained from donors having either a wildtype or a mutant MBL genotype for their ability to activate C4. Our data indicate that sera from donors having heterozygous or homozygous mutations in the first exon of the MBL gene are partially deficient in activation of C4 both by IgA and by mannan. Since C4 activation by the classical pathway is similar in both groups, the observed
differences in mannan and IgA-induced C4 activation can not be based on differences in the classical pathway activity or the concentration of active C4. Therefore, these data are strongly suggestive for the involvement of the lectin pathway in C4 activation by IgA in whole serum.

Complement activation by IgA has been subject of investigation already during several decades. It is generally agreed that IgA cannot activate the classical complement pathway (14). Activation of the alternative complement pathway by IgA is supported by both in vitro (27-29) and in vivo observations (30), as well as by the present study. It has been argued that complement activation by IgA has to rely on studies using artificially modified or presented IgA (14). However, complement activation has also been demonstrated upon binding of IgA to its natural antigen. For example, xenoreactive human IgA antibodies can induce complement-mediated lysis of pig endothelial cells in a calcium-independent way (28). Furthermore, binding of human serum IgA to Streptococcus pneumonia induces neutrophil-mediated bacterial killing which was complement-dependent and proceeded in the presence of MgEGTA (29).

A strong suggestion for the activation of complement by human IgA in vivo is present in patients with IgA nephropathy. IgA nephropathy is a common glomerular disease characterized by mesangial deposition of IgA and complement components (31). Furthermore, deposition of C4 and C4-binding protein was shown in 30% and 60% of cases, respectively, whereas only 6% showed the presence of C1q (31). Alternative pathway activation by IgA may explain the deposition of C3 but not that of C4 in IgA nephropathy. Therefore, activation of C4 by IgA via the lectin pathway, as demonstrated in the present study, may very well be the mechanism of C4 activation in IgA nephropathy. This hypothesis is strongly supported by the deposition of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19; 20) and patients with Henoch-Schönlein purpura (32). The latter disease is also characterized by mesangial deposition of IgA and complement.

Lectin pathway activation by IgA was most prominent for polymeric IgA, followed by dimeric IgA and monomeric IgA. Similar differences have been previously reported for activation of the alternative pathway by rat and human IgA (16; 26; 29). In addition, polymeric IgA shows enhanced binding to the phagocytic IgA Fc receptor CD89 (33) and to human mesangial cells (34). The stronger effector functions of polymeric IgA have a beneficial role for the defense functions of IgA (29; 35; 36). In accordance, circulating antigen-specific IgA produced upon primary pathogen contact predominantly consists of polymers (29). The polymeric nature of mesangial IgA in IgA nephropathy (37) will most likely contribute to the development of renal damage, involving complement activation and mesangial cell activation.
At present it is unknown which part of the IgA molecule is involved in binding to the CRD of MBL. IgA is a heavily glycosylated molecule (reviewed in (15)). Several variants in the sugar composition have been described, among which high mannose type N-linked glycan chains (38). Especially the latter glycosylation variant may be a likely candidate to serve as a ligand for MBL. Interestingly, circulatory IgA in patients with IgA nephropathy shows an abnormal glycosylation, characterized by a decreased galactosylation of O-linked sugar chains (39). Patients with rheumatoid arthritis produce increased levels of a certain glycoform of IgG that lacks the terminal galactose moieties on the N-terminal glycan chains. This so-called G0-IgG has been shown to bind MBL (40). The hypothesis that altered glycosylation of IgA contributes to complement activation in IgA nephropathy is presently under investigation.

Binding of IgA to microorganisms enables its interaction with phagocytes via the phagocytic Fcα receptor CD89 (29). Together with complement activation, this may result in pathogen elimination, involving CD89 and complement receptors. MBL binding may directly contribute to phagocytosis via MBL receptors (41; 42). In this respect it is conceivable that complement receptors and IgA receptors act together in pathogen elimination (29). In a similar way, classical pathway activation via IgG antibodies works in concert with Fcγ receptors (43).

Based on a number of studies in individuals with MBL gene mutations, the prominent role of MBL in innate immunity has been well appreciated. The protective role of MBL against infection can be explained by the direct binding of MBL to microorganisms (1; 13). MBL binding to IgA may be an additional protective mechanism against microorganisms to which MBL does not bind directly. In situations in which pre-exposure to a pathogen has taken place, such as after vaccination or during chronic infection, MBL may act in concert with IgA to maintain host integrity. Such a mechanism is conceivable for example in cystic fibrosis, in which chronic lung infections often lead to irreversible pulmonary damage and death. Expression of MBL variant alleles in patients with cystic fibrosis is associated with a severely reduced life span (12).

Although MBL is an important defense factor of the immune system, it may also play an unfavorable role in disease progression. This is proposed for rheumatoid arthritis, and is suggested by the presence of MBL in renal biopsies from patients with IgA nephropathy, Henoch-Schönlein purpura, SLE, and post-streptococcal glomerulonephritis (19; 20; 32; 40; 44). IgA nephropathy is the leading cause of end stage renal disease worldwide. Therefore, the IgA-binding function of MBL described in the present study is also likely to play a dual role in immunity. On the one hand, it may link the innate and the adaptive immune system and thereby protect the individual against invading pathogens. In this respect, antibody-mediated complement activation can be considered as an additional parallel between MBL and Clq. On
the other hand, it may enhance the pro-inflammatory effects of IgA deposition in the glomerulus, ultimately leading to renal injury.

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