Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2

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Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2

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Introduction
Mannan-binding lectin (MBL; also referred to as mannose-binding lectin) is a Ca2+-dependent lectin belonging to the collectin family of proteins and is an important constituent of innate immunity (1–3). The protein is an oligomer of subunits that each contains 3 identical polypeptide chains with a C-terminal carbohydrate recognition domain, a neck region, a collagenous region, and an N-terminal cystein-rich region. MBL selectively recognizes patterns of sugars such as mannose, N-acetylgalactosamine, and fucose on microbial surfaces. MBL oligomers form complexes with 3 proteases, MBL-associated serine protease 1 (MASP-1; ref. 4), MASP-2 (5), and MASP-3 (6), and nonenzymatic protein MAP19 (7). Low-order MBL oligomers mainly associate with MASP-1 and MAP19, while higher oligomers associate with MASP-2 and MASP-3 (6). L-ficolin and H-ficolin are 2 other recognition proteins that form complexes with MASPs (3, 8). Binding of MBL/MASP or ficolin/MASP complexes to target molecules activates the lectin pathway of complement with recruitment of C3 and the terminal complement components (C5–C9) (8, 9). Thus complement activation can proceed through 3 major pathways, the classical pathway (C1qC4C2, C4, C2, and C3), the alternative pathway (C3, factor B [B], factor D [D], and properdin [P]), and the lectin pathway (10). MASP-1 and MASP-2 are controlled by C1 inhibitor (C1 INH) and may also be controlled by α2-macroglobulin (11–13). Complement activation through the lectin pathway is known to involve activation of MASP-2, which then cleaves C4 and C2 with formation of the classical pathway C3 convertase (C4b2a) (5, 14). Activation of C3 by C4b2a is amplified through recruitment of the alternative pathway (9). A second mechanism for MBL pathway–dependent activation of C3 and the alternative pathway has been suggested to be based on direct C3 cleavage by MASP-1 (15). However, C3 cleavage by MASP-1 is inefficient, which has cast doubt upon the biological role of a C4b2a-independent mechanism (11–13). The question concerning the existence of a functional MBL-dependent bypass mechanism is of considerable interest and may be clinically relevant in conditions such as C4 and C2 deficiency.

Homozygous deficiencies of C4 and C2 are genetically well-defined immunodeficiency states that predispose to infection and development of immunological disease (16, 17). C2 deficiency has an estimated prevalence of about 1 in 20,000 in Western populations (18). Complete C2 deficiency, C2 deficiency type I, is usually caused by a 28-bp deletion of the C2 gene, while C4 deficiencies are very rare and have a more heterogeneous genetic background (16, 18). Most complement functions in C2 or C4 deficiency rely on an intact alternative pathway. MBL deficiency, which has a broad, less distinct spectrum of disease associations compared with other complement deficiencies, is remarkably common and has a prevalence of more than 5% in several populations (1, 3). It is caused by homozygosity or compound heterozygosity involving structural gene mutations at...
Table 1

<table>
<thead>
<tr>
<th>Serum</th>
<th>MBLα (mg/l)</th>
<th>Anti-CO IgM (mg/l)</th>
<th>Anti-CO IgG (mg/l)</th>
<th>MBL genotype</th>
<th>Bδ (mg/l)</th>
<th>Pδ (mg/l)</th>
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<tr>
<td>PNHS</td>
<td>1.75</td>
<td>0.91</td>
<td>0.82</td>
<td>–</td>
<td>200</td>
<td>25</td>
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<tr>
<td>MBLD</td>
<td>&lt; 0.015</td>
<td>5.56</td>
<td>8.7</td>
<td>–</td>
<td>158</td>
<td>23</td>
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<tr>
<td>C1qDP-depleted MBLD</td>
<td>&lt; 0.015</td>
<td>4.1</td>
<td>8.3</td>
<td>–</td>
<td>150</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>C2D:9</td>
<td>0.44</td>
<td>0.28</td>
<td>&lt; 0.05</td>
<td>AHY/BLY</td>
<td>174</td>
<td>36</td>
</tr>
<tr>
<td>C2D:17</td>
<td>2.72</td>
<td>0.48</td>
<td>&lt; 0.05</td>
<td>AHY/ALX</td>
<td>110</td>
<td>22</td>
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<tr>
<td>C2D:18</td>
<td>2.69</td>
<td>0.41</td>
<td>&lt; 0.05</td>
<td>AHY/AHY</td>
<td>144</td>
<td>28</td>
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<tr>
<td>C2D:21</td>
<td>0.051</td>
<td>&lt; 0.1</td>
<td>&lt; 0.05</td>
<td>ALX/BLY</td>
<td>132</td>
<td>21</td>
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<tr>
<td>C4D</td>
<td>10.0</td>
<td>0.23</td>
<td>&lt; 0.05</td>
<td>AHY/AHY</td>
<td>382</td>
<td>33</td>
</tr>
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</table>

MBLα, MBL-deficient serum; αReference interval, 0.1–6.0 mg/l. βReference interval, 118–308 mg/l. γReference interval, 13.5–39 mg/l.

In the present study, the capacity to support C3 deposition was studied by ELISA in a panel of sera with defined defects of complement function. In the ELISA, surface-adsorbed polyacrylamide-conjugated O antigen–specific oligosaccharides (21) derived from Salmonella typhimurium (serogroup B), S. thompson (serogroup C), and S. enteritidis (serogroup D) were used as antigens. Complement-activating properties of the 3 oligosaccharides have been previously studied (22), and Salmonella serogroup C has been reported to bind MBL (23). C2 deficiency type I sera (24) were investigated together with MBL-deficient sera and sera with combined C2 and MBL deficiency (25), C4-deficient serum (26), and sera that were specifically depleted of classical and alternative pathway components (27) and of MASP. The sera were reconstituted with purified proteins in different combinations for selective analysis of complement pathways. Calibrated methods were used for quantitation of antibodies to the Salmonella O antigens (25). The results demonstrated that C3 and the alternative pathway were activated through an MBL-dependent C2 bypass mechanism. The mechanism may be biologically important and did not appear to require recruitment of MASP-1, MASP-2, and MASP-3.

Results

C3 deposition induced by Salmonella O antigen–specific oligosaccharides. C3 deposition was assessed in pooled normal human serum (PNHS) and in serum from C2-deficient patient 18 (C2D:18; Table 1) using different amounts of polyacrylamide conjugate of Salmonella serogroup B–specific oligosaccharide (BO), C–specific oligosaccharide (CO), and D–specific oligosaccharide (DO) for coating. At coating doses of 100 and 1,000 ng/well, the CO antigen gave pronounced C3 deposition in both sera (Figure 1, A and B). The BO and DO antigens produced very little C3 deposition in C2-deficient serum but were moderately active in PNHS. Kinetic C3 deposition experiments with PNHS and the C2-deficient sera C2D:9, C2D:17, and C2D:18 confirmed that C3 deposition was much more pronounced in the presence of CO antigen than in the presence of BO and DO, particularly in C2-deficient serum (Figure 1, C and D).

Requirement of MBL and other recognition molecules. In order to examine MBL binding in our assay system, different dilutions of MBL-sufficient serum were added to wells coated with BO, CO, or DO followed by measurement of bound MBL. In Ca2+-containing Veronal-buffered saline (VBS), MBL binding to CO was readily detectable at concentrations down to 0.005 mg/l. MBL did not bind to BO and DO even at high MBL concentrations (2 mg/l). Thus, MBL bound selectively to the CO antigen. No MBL binding occurred in the presence of VBS-EDTA (data not shown).

We then examined whether serum supported C3 deposition on CO antigen without involvement of MBL or Igs. An MBL-deficient serum with high concentrations of IgM and IgG antibodies to CO (Table 1) efficiently supported C3 deposition, which could be ascribed to classical pathway activation (28). This MBL-deficient serum was depleted of C1q, D, and P (C1qDP-depleted) as well as
Igs (see Methods). CO antigen did not induce C3 deposition when the alternative pathway was reconstituted by addition of D and P in the absence of MBL and Igs (Figure 2). Moreover, addition of C1q in different combinations had no effect (data not shown). Thus specific recognition molecules were necessary.

With addition of MBL/MASP to the serum used, lectin pathway activation resulting in C3 deposition could be mediated through C4b2a as well as through C2-independent mechanisms. Dose-dependent C3 deposition was demonstrated when MBL/MASP was added at increasing concentrations (Figure 2), and addition of MBL/MASP plus D and P allowed activation and amplification through the alternative pathway. The impact of the alternative pathway was evident at moderately low MBL/MASP concentrations (0.2 mg/l). Most likely, this reflected amplification of C4b2a-mediated C3 activation. At high MBL/MASP concentrations (2 and 20 mg/l), amplification was not required in order to obtain pronounced C3 deposition. The assay did not allow assessment of amplification at this level.

No C3 deposition was found when the experiment was repeated in VBS-Mg²⁺EGTA with addition of MBL/MASP to the serum at 2 mg/l. We also presensitized the CO-coated wells with MBL/MASP at 2 mg/l in VBS and in VBS-EDTA. After washing, the wells were incubated with MBL-deficient serum depleted of C1qDP as well as Igs. No C3 deposition occurred unless the presensitization step was carried out in the presence of Ca²⁺ (data not shown).

We then examined the potential role of specific antibodies in the MBL-deficient serum. For this purpose, CO-coated wells were presensitized with MBL-deficient serum that was depleted of C1qDP but not of Igs. The serum was diluted in VBS or VBS-EDTA to reach a concentration of 20%, the serum concentration used for subsequent assessment of C3 deposition. After washing, MBL-deficient serum depleted of C1qDP and Igs was added in combination with purified complement proteins. Addition of C1q or addition of D and P produced efficient C3 deposition, showing that the classical pathway and the alternative pathway were both capable of supporting antibody-dependent activation. No C3 deposition occurred when the presensitization step was omitted. Identical results were obtained following presensitization in VBS and presensitization in VBS-EDTA (data not shown).

**Complement pathway requirements.** The kinetic C3 deposition experiments with PNHS and C2-deficient serum (Figure 1, C and D) were repeated in Mg²⁺EGTA buffer with the purpose of blocking Ca²⁺-dependent binding of MBL to the CO antigen with retention of alternative pathway function. C3 deposition was reduced to the level found with BO- or DO-coated wells (data not shown).

**Figure 2**
C3 deposition induced by CO antigen (1,000 ng/well) in an MBL-deficient serum that was depleted of C1qDP and Igs. The serum was used at a final concentration of 20% and was studied with or without reconstitution of the alternative pathway. Reconstitution of undiluted serum with C1q (70 mg/l, not shown) or D (1 mg/l) together with P (25 mg/l) did not promote C3 deposition, showing that the CO antigen did not activate complement in the absence of recognition proteins. Addition of MBL/MASP complexes promoted C3 deposition in a dose-dependent manner; alternative pathway-mediated amplification was evident at moderately low MBL/MASP concentrations but was not required at high MBL/MASP concentrations. C3 deposition was measured after 60 minutes at 37°C. The experiment was performed in duplicate and was repeated once with similar results.

**Figure 3**
Complement requirements for C3 deposition induced by CO antigen (1,000 ng/well). Analysis of MBL-deficient serum (A) and C2-deficient, MBL-sufficient serum (B). The sera were C1qDP depleted and reconstituted with C1q (70 mg/l), D (1 mg/l), and P (25 mg/l) added alone or in combinations. Sera were used at a final concentration of 25%. Interpretations are shown at top. Each experiment was performed in duplicate and was repeated once with similar results.
shown), providing further evidence for the critical role of MBL binding in the assay.

The kinetic studies were continued using MBL-deficient serum and C2D:18 that were both C1qDP depleted. C2D:18 contained high concentrations of MBL and some antibody to CO (Table 1). The capacity of the sera to support C3 deposition in the presence of CO was assessed after substitution with purified C1q, D, and P added alone or in combinations. Addition of C1q alone to the MBL-deficient, C1qDP-depleted serum fully restored the capacity to support C3 deposition, demonstrating classical pathway activation in the absence of a functional alternative pathway (Figure 3A). Selective reconstitution of the alternative pathway by addition of D and P resulted in C3 deposition at a slow rate. We ascribed the efficient classical pathway–mediated activation to the presence of specific antibodies (Table 1). In addition, the antibodies supported alternative pathway activation. It is noteworthy that the alternative pathway–mediated response required a CO antigen coating dose of 1,000 ng/well and was not seen with a coating dose of 100 ng/well (data not shown).

Results with C2-deficient serum differed sharply from those obtained with MBL-deficient serum. In the C1qDP-depleted, C2-deficient serum, C3 deposition was equally well restored by D and P and by C1q, D, and P (Figure 3B). As the serum was MBL sufficient (Table 1), these findings suggested C3 activation by an MBL-dependent C2 bypass mechanism with requirement of intact alternative pathway function. Addition of C1q alone had no effect, but a combination of C1q and D produced slow C3 deposition in the serum. In this case, C3 activation probably proceeded through the well-known C1q-dependent C2 bypass mechanism triggered by antibodies (29).

Influence of MBL and antibody concentrations in C2-deficient sera. C3 deposition in wells coated with CO antigen was assessed in the sera of 21 patients with C2 deficiency. The capacity to support C3 deposition in the sera was correlated with concentrations of MBL ($P < 0.05$, Spearman rank correlation; Figure 4) but not with the concentrations of specific antibodies (data not shown). Although weak, the correlation indicated that MBL is a major determinant of C3 activation in the assay system. Many factors, including alternative pathway function, are likely to influence the results. C4-deficient serum with a high concentration of MBL (10 mg/l) also supported efficient C3 deposition (mean absorbance, 1.4; data not shown). This was probably due to combined effects of high MBL and B concentrations (Table 1).

Of the 3 C2-deficient sera with undetectable MBL (<0.015 mg/l), 2 showed moderately high C3 deposition that could not be readily explained by antibody levels. One C2-deficient patient, C2D:21, demonstrated low concentrations of MBL and anti-CO antibodies (Table 1) and low C3 deposition. This serum was utilized in several of the subsequent experiments.

Reconstitution experiments in serum with combined C2 and MBL deficiency. The serum of C2D:21 provided a useful tool for reconstitution experiments. First, the influence of purified MBL and MBL/MAST complexes on C3 deposition onto solid-phase CO antigen was examined. MBL and MBL/MAST complexes were added to the serum or were used for presensitization of CO-coated wells before addition of serum.
Presensitization of CO-coated wells with MBL at the physiological doses of 0.2 and 2.0 mg/l clearly promoted C3 deposition (Figure 5A). The effect was further enhanced with MBL at 20 mg/l. Similar results were obtained when MBL was added directly to serum before analysis. Addition of MBL/MASP complexes at 2–20 mg/l to serum resulted in pronounced C3 deposition that proceeded at a comparatively fast rate (Figure 5B). Presensitization experiments with MBL/MASP gave similar results (data not shown).

We also examined the effect of specific anti-CO and anti-BO antibodies in a preparation of polyclonal IgG (Endobulin). Microtiter plate wells coated with CO or BO antigen were presensitized with defined doses of specific IgG in VBS-EDTA. After washing of the wells, C3 deposition was studied in C2D:21 serum. Anti-CO antibodies supported C3 deposition in a dose-dependent manner (Figure 5C), but anti-BO antibodies had no effect. Of note, no functional MBL was detected in the Ig preparation.

We then examined the effects of serum dilution on C3 deposition in the presence of CO (Figure 6). Addition of MBL or C2 to C2D:21 serum promoted C3 deposition in 20% and 40% serum, but effects in more dilute serum were negligible. By contrast, the highest C3 deposition values in PNHS were obtained using 5% serum. Similar findings were made when C2D:21 serum was reconstituted with a combination of MBL (2 mg/l) and C2 (6.5 mg/l). Reconstitution of the serum with C2 had a very modest effect, indicating that the contribution of antibody-mediated C3 activation was limited and that the small amount of MBL in the serum did not efficiently support C4b2a-mediated C3 deposition through the lectin pathway.

Reconstitution with fractionated MBL/MASP complexes and MBL. Purified MBL/MASP complexes were fractionated according to charge and size by chromatography on a Mono Q column (6). Aliquots of the fractions (1 μl) were added to C2D:21 serum (12.5 μl) in a total volume of 0.05 ml for analysis of their capacity to promote C3 deposition on CO-coated wells. C3 deposition was determined with fractions corresponding to the position of low-order MBL oligomers (6) and MASP-1 in the first part of the chromatogram (Figure 7).

Reconstitution of C2D:21 serum with C2 before addition of the fractionated MBL/MASP complexes promoted C3 deposition, and the capacity of the fractions to support C3 deposition then coincided with the whole peak of fractionated MBL/MASP complexes (Figure 7). The peak concentration of eluted MBL (fraction 37) was calculated to yield a final MBL concentration of 1.44 μg/ml in undiluted C2D:21 serum. Thus MBL/MASP complexes were added to serum at fairly modest concentrations.

MBL-dependent C3 deposition in the absence of C2 and MASPs. The MBL-deficient serum of C2D:21 was depleted of C1qDP and serum were negligible. By contrast, the highest C3 deposition values in PNHS were obtained using 5% serum. Similar findings were made when C2D:21 serum was reconstituted with a combination of MBL (2 mg/l) and C2 (6.5 mg/l). Reconstitution of the serum with C2 had a very modest effect, indicating that the contribution of antibody-mediated C3 activation was limited and that the small amount of MBL in the serum did not efficiently support C4b2a-mediated C3 deposition through the lectin pathway.

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MBL-dependent C3 deposition in the absence of C2 and MASPs. The MBL-deficient serum of C2D:21 was depleted of C1qDP and
MASPs (see Methods). Reconstitution experiments with recombinant MBL (rMBL) D, and P indicated that CO-induced C3 deposition required rMBL and a functional alternative pathway and that the activity was independent of MASP-1, -2, and -3 (Table 2). A confirmatory experiment was performed with the serum of C2D:17. Control experiments using ELISA plates coated with BO or DO antigen gave no C3 deposition, providing evidence for assay specificity and showing that target binding of rMBL was necessary.

**MBL-dependent C2 bypass activation by other microbial carbohydrates.** C3 deposition in the serum of C2D:21 was examined using mannan-coated ELISA plates and plates coated with mannan-rich LPS from *E. coli* O8, O44, and O77 strains. In contrast to CO, these carbohydrates were efficient activators of the alternative pathway. Addition of rMBL consistently increased the kinetics of C3 deposition (Figure 8). Partial delipidation of LPS appeared to promote MBL-dependent C2 bypass activation. Similar results were obtained with LPS from *E. coli* O44 and O77 (data not shown).

**Discussion**

O antigen–specific *Salmonella* oligosaccharides are well-defined microbial antigens (21, 22) and were used as solid-phase targets in an ELISA model system for analysis of C3 deposition in serum. CO supported complement activation by several mechanisms, including MBL-dependent recruitment of C4b2a through the lectin pathway and antibody-mediated activation of the classical pathway. This is consistent with the recent suggestion that antibody-mediated classical pathway activation partly compensates for MBL deficiency as assessed by studies of mannan antibodies (28). In addition, our analysis provided evidence of alternative pathway–mediated activation of C3 by specific antibodies and by the C1q-dependent C2 bypass mechanism (29). Polymeric IgA has recently been suggested to activate the lectin pathway (30) and might also have contributed to activation.

The principal finding of the study was the demonstration of a functional MBL-dependent C2 bypass mechanism for C3 activation. The C2 bypass mechanism required a serum concentration of at least 20% and intact alternative pathway function. C4 did not appear to be required. Unlike CO, many natural activators of the lectin pathway may be assumed to activate the alternative pathway. The present findings showed that activation of the MBL-dependent C2 bypass by solid-phase mannan or mannan-rich LPS of *E. coli* markedly increased the kinetics of C3 deposition, which might be highly significant in the course of inflammatory responses.

In more dilute serum, MBL-dependent activation was mediated by C4b2a and did not involve the alternative pathway. A recently described lectin pathway assay using mannan-coated ELISA plates is strictly C4b2a dependent (31). Under physiological conditions, C4b2a-mediated cleavage of C3 with amplification through the alternative pathway is likely to be the predominant mechanism for MBL-dependent complement activation (9). Dose-response experiments demonstrated the importance of alternative pathway–mediated amplification at moderately low MBL concentrations.

The possible contribution of MASP complexes to MBL-dependent C2 bypass activation of C3 is a controversial issue. We provided evidence that MASP-1, MASP-2, and MASP-3 are not necessary for C2 bypass activation of C3, in accord with previous data showing that cleavage of purified C3 by MASP-3 is inefficient (13). Moreover, results of fractionation experiments with purified MBL/MAST complexes argued against a role of MASP-2, since activation of the alternative pathway was mediated by C4b2a and did not involve the alternative pathway. The present findings showed that activation of the MBL-dependent C2 bypass by solid-phase mannan or mannan-rich LPS of *E. coli* markedly increased the kinetics of C3 deposition, which might be highly significant in the course of inflammatory responses.

Our findings suggested that target-fixed MBL activates the alternative pathway, which we believe to be a novel observation. Thus bound MBL probably provides C3b binding sites that are protected from inactivation by factors H and I, as previously described for bound IgG (32) and for C4 (33). The question may be raised as to whether C1 INH releases activated MASP complexes from target-fixed MBL, thereby promoting alternative pathway activation. There is some evidence for C1 INH–dependent dissociation of MBL/MAST complexes (34), but this issue has not been fully investigated. C1qS2 complexes are known to be dissociated by C1 INH in conjunction with activation (35, 36). C3 and C4 form complexes with C1q (37), indicating a potential for C1q-mediated activation of the alternative pathway.

It is not known whether the C3 deposition following addition of MBL/MAST to serum (Figure 5B) required complex dissociation that may have delayed responses. Evidently, the kinetics in many of the experiments were slow compared with the kinetics of functions such as alternative pathway–mediated hemolysis at similar serum concentrations.
MASP complexes and were then investigated for D were investigated. The bacteria were presensitized with MBL/sheep erythrocytes coated with CO produce efficient C3 break

L-ficolin and H-ficolin structurally resemble MBL and bind carbohydrates such as N-acetylglucosamine (3, 8). Moreover, L-ficolin was recently shown to be specific for acetyl groups (39). Both recognition proteins form complexes with MASPs and activate the lectin pathway (3, 8). The possibility that ficolins are capable of recognition proteins form complexes with MASPs and activate the classical pathway as well as the alternative pathway is involved in complement activation by LPS, with the polysaccharide part being responsible for alternative pathway activation (42). The present findings emphasize the role of the lectin pathway in antibody-independent complement activation by LPS.

An interesting question concerns the possible impact of MBL in complement deficiency states. In inherited C4 and C2 deficiencies, concomitant MBL deficiency implies loss of direct MBL-mediated opsonization (43) as well as an impaired capacity to recruit complement. Considerations of this kind may also be relevant in acquired deficiencies of C4 and C2 such as SLE (44). Increased susceptibility to infection has been reported in SLE patients with MBL deficiency (45). MBL-dependent functions might also be important in MASP-2 deficiency, the most recently recognized form of complement deficiency (46).

**Methods**

Buffers. The buffers used for this study were as follows: VBS with 0.15 mmol/l Ca$^{2+}$ and 0.5 mmol/l Mg$^{2+}$ (VBS); VBS with 10 mmol/l EDTA (VBS-EDTA); VBS with 10 mmol/l EGTA and 4 mmol/l Mg$^{2+}$ (VBS-Mg$^{2+}$ EDTA); VBS with 0.1% gelatine (VBSG); PBS with 5 mmol/l EDTA, pH 7.2 (PBS-EDTA); and PBS with 0.05% Tween (PBS-T).

Oligosaccharide conjugates. Polyacrylamide conjugates of S. typhimurium (serogroup B; O4,12) SH 4809 octasaccharide, S. thompson (serogroup C; O6,7) IS 40 decasaccharide, and S. enteritidis (serogroup D; O9,12) SH 1262 octasaccharide were prepared as described by Chernyak et al. (21).

Other carbohydrates. Mannan-coated ELISA plates were kindly provided by Wieslab AB. We also examined LPS from 3 mannose-rich E. coli strains, E. coli O8 (47), E. coli O44 (48), and E. coli O77 (49).

Partially delipidated LPS (LPS-OH) was prepared by de-O-acylation of ester-linked fatty acids from the lipid A with 0.25 M NaOH at 37 °C for 16 hours. After centrifugation, pH was adjusted to 3.5, and free fatty acids were removed by repeated extractions with CHCl$_3$. After adjustment of pH to 7.0, the LPS-OH was extensively dialyzed against distilled water (48 hours at 4°C) and lyophilized.

Purified proteins. MBL, MBL/MASP complexes, and C2 were isolated according to previously described procedures (6, 11), as were C1q, D, and P (27) and C3 (50). MBL/MASP complexes were further fractionated by ion exchange chromatography on a Mono Q column (Amersham Pharmacia) as previously described (6). The rMBL used in some of the experiments sources were used at concentrations (2%) that would not normally support alternative pathway activation.

Interactions between LPS and the immune system have been intensely studied over the years. Cell-wall LPS from wild-type serum-resistant Gram-negative enteric bacteria such as Salmonella is structurally and functionally composed of 3 parts: the toxic lipid A region, the common core polysaccharide, and the O antigen–specific polysaccharide (40). With regard to complement, Pillemer et al. (41) originally suggested that LPS activates the properdin system, now known as the alternative pathway, without involvement of antibodies. It was later shown that the classical pathway as well as the alternative pathway is involved in complement activation by LPS, with the polysaccharide part being responsible for alternative pathway activation (42). The present findings emphasize the role of the lectin pathway in antibody-independent complement activation by LPS.
was produced as previously described (51). Complement proteins were mostly determined by electroimmunoassay (52). In some experiments, C2 was measured by hemolytic assay (53). Concentrations of C1q, D, P, B, C2, and C3 were given in mg/l assuming that the pooled reference serum used contained C1q at 70 mg/l, D at 1 mg/l, P at 25 mg/l, B at 200 mg/l, and C2 at 26 mg/l (54). A commercial calibrator (Dako) was used for Ig measurements. Human purified IgG, Endobulin (Baxter), was used as source of specific anti-BO and anti-CO antibodies and was a gift from Immuno.

MASP-1 and MASP-3 were measured by ELISA using a monoclonal antibody against the common heavy chain of the proteins (55). MASP-2 was also determined by ELISA (56).

Measurement of specific antibodies and MBL. Specific IgM and IgG antibodies to BO, CO, and DO were measured by ELISA according to a procedure that allowed expression of the concentrations in mg/l (25). IgA antibodies were rarely found. MBL concentrations were determined with a sandwich ELISA (57), in which microtiter plates (Nunc-Immunoplates Maxisorp; Nunc) were coated with mouse monoclonal anti-MBL (clone 131-1, IgG1κ; Immunonex Therapeutics). Control wells were coated with another mouse IgG1κ monoclonal antibody. Biotinylated mouse monoclonal anti-MBL (Immunonex Therapeutics) followed by enzyme-labeled streptavidin (Streptavidine AP, D0936; Dako) was used for detection. Values were given in weight units using an MBL standard serum for calibration (Immunonex Therapeutics). In some experiments, biotinylated mouse monoclonal anti-MBL was used together with enzyme-labeled streptavidin for measurement of MBL binding to ELISA microtiter plate wells coated with BO, CO, or DO.

MBL genotyping. Analysis of MBL haplotypes was performed largely as described by Steffeens et al. (19), with identification of the structural wild-type allele A, the mutant alleles B, C, and D, and the promoter region haplotypes HY, LY, and LX.

Serum and serum reagents. All sera were stored in aliquots at −80°C. The complement-deficient sera used in the study included sera from 21 patients with C2 deficiency type 1 (24) and 1 patient with complete C4 deficiency (26). Blood sampling was performed after informed consent was provided, within the framework of projects approved by the Lund University Research Ethics Committee (LU 350-93, LU 513-01). PNHS from 17 healthy blood donors was used as a control. A unit of fresh frozen serum, purchased from the Blood Bank of the University Hospital of Lund, was incidentally found to be MBL deficient (MBL <0.015 mg/l). The MBL deficiency was confirmed (MBL 0.005 mg/l) by time-resolved immunofluorometric assay (58).

Sera from previously reported (24) C2-deficient patients C2D:17, C2D:18, and C2D:21 and the MBL-deficient serum were depleted of the complement proteins C1q, D, and P (27). The C1qDP-depleted, MBL-deficient serum was further depleted of IgG by affinity chromatography using protein LG-Sepharose (58) followed by affinity chromatography using rabbit anti-α and anti-λ antibodies (Dako) coupled to cyanogen bromide-activated (CNBr-activated) Sepharose 4B (Amersham Biosciences). The concentrations of IgG, IgA, and IgM in the Ig- and C1qDP-depleted MBL-deficient serum were less than 20 mg/l, 5 mg/l, and 30 mg/l, respectively, as determined by immunochromehemical methods. Concentrations in the sera of MBL, IgM anti-CO antibodies, and IgG anti-CO antibodies as well as the MBL genotypes of the donors are summarized in Table 1.

C2D:21 had a combined C2 and MBL deficiency (Table 1). After C1qDP depletion, the serum was further depleted of MASP. For immunoadsorption of MASP-1 and MASP-3, the monoclonal antibodies 1E2 and 2B11 (11, 55) and 2D3 were coupled to CNBr-activated Sepharose beads (Amersham Biosciences). Monoclonal antibodies to MASP-2 (56) were coupled to CNBr-activated Sepharose in the same fashion. Control beads with mouse IgG were also prepared. Immunoadsorptions were carried out according to a batch-wise procedure, wherein 1 ml of C1qDP-depleted C2D:21 serum in VBS was added to an equal volume of sedimented Sepharose beads in sealed tubes that were rotated end over end at 4°C for 18 hours. After sedimentation of the beads, the immunoadsorbed serum was collected. The serum was centrifuged at 2,000 g for 15 minutes to remove remaining particles and was then frozen at −80°C. The beads were washed and were then eluted with Trit/HCl, pH 2.8. After single immunoadsorption the sera contained less than about 2% of the original MASP-3 and MASP-2 concentrations. One aliquot of C1qDP-depleted C2D:21 serum was immunoadsorbed twice in order to ensure complete removal of MASP-1 and MASP-3, and another aliquot was immunoadsorbed twice to remove MASP-2. A third aliquot was immunoadsorbed once with anti-MASP-1 and anti-MASP-3 Sepharose and once with anti-MASP-2 Sepharose. Treatment of serum with control Sepharose beads coupled to mouse IgG did not influence MASP concentrations.

To confirm results with C2D:21 serum, the C1qDP-depleted serum of C2D:17 (Table 1) was also depleted of MASP-1, -2, and -3 and of MBL. Aliquots of the serum (1 ml) in VBS at 4°C were sequentially passed through three 1-ml affinity chromatography columns containing (a) anti-MASP-1 and anti-MASP-3 Sepharose, (b) anti-MASP-2 Sepharose, and (c) mannan-Sepharose (11). The pooled preparations contained trace amounts of MASP-3 (1–2%). For this reason, immunoadsorption with anti-MASP-1 and anti-MASP-3 Sepharose was repeated once using the batch-wise procedure.

C3 deposition assay. C3 deposition was assessed by ELISA. Microtiter plates (Nunc-Immunoplates Maxisorp; Nunc) were coated with BO, CO, or DO antigen in 50 mmol/l carbonate buffer, pH 9.5, overnight at 4°C. The coating dose was 1.000 ng/well unless otherwise specified. Three wells were used for each sample, 2 coated wells and 1 uncoated well. After washing with PBS, all wells were blocked with VBSG for 2 hours at room temperature. After washing with PBS and VBS, sera diluted in VBS were added to each of the 3 wells at 0.05 ml/well. The plates were incubated at 37°C in a thermoblock (Techne) for 15–60 minutes and were then washed with PBS-T. Rabbits anti-C3c antibodies (Dako) conjugated (59) with alkaline phosphatase (Type VII-T; Sigma-Aldrich) were added at 0.05 ml/well. This antibody has previously been shown to react with C3, C3b, iC3b, and C3c, but not with C3d/g in ELISA and immunoblotting systems (60). After incubation at room temperature for 2 hours, the plates were washed with PBS-T. The reaction was visualized by adding 0.05 ml/well of p-nitrophenylphosphate (1 g/l; Sigma-Aldrich) in 10% diethanolamine, pH 9.8. After 30 minutes’ incubation in the dark at room temperature, the enzymatic reaction was measured at 405 nm in a Multiskan Plus photometer (Labsystems). Control sera were included for adjustment of interassay variation. C3 deposition was expressed as the mean absorbance in the duplicate oligosaccharide-coated wells with subtraction of the background absorbance in the uncoated well. In a few supplemental experiments, the C3 protein remaining in supernatants of oligosaccharide-coated wells and control wells was measured by electroimmunoassay in order to further assess C3 deposition.

Reconstitution experiments. In the reconstitution experiments, the concentrations of purified proteins added were expressed according to the concentrations in undiluted serum. When a single dose of purified protein was used, the concentration usually approximated the mean physiological concentration (100%). In some experiments C1q and C2 were used at lower concentrations (10–20% of normal) based on titrations indicating that these concentrations were not limiting in the assays. When complement-depleted sera were used, the dilutions were adjusted so as to achieve the serum protein concentrations obtained with diluted whole serum. In some experiments, reconstitution of function was achieved by presensitization procedures. For presensitization of oligosaccharide-coated ELISA plate
wells, recognition proteins (MBL or antibodies) were added to the wells for 30–60 minutes at room temperature in Ca²⁺-containing VBS or VBS-EDTA. After extensive washing of the wells, serum reagents were added for investigation of C3 deposition.

**Statistics.** Correlation was assessed with the Spearman rank correlation test. A P value less than 0.05 was considered significant.

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