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Activation of Complement by Mannose-Binding Lectin on Isogenic Mutants of Neisseria meningitidis Serogroup B

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Mannose-binding lectin (MBL) is a serum protein that has been demonstrated to activate the classical complement pathway and to function directly as an opsonin. Although MBL deficiency is associated with a common opsonic defect and a predisposition to infection, the role of the protein in bacterial infection remains unclear. We have investigated MBL binding to Neisseria meningitidis serogroup B1940 and three isogenic mutants, and the subsequent activation of the two major isoforms of C4 (C4A and C4B) by an associated serine protease, MASP. The mutants lacked expression of the capsular polysaccharide (siaD\(^2\)), the lipo-oligosaccharide (LOS) outer core that prevented LOS sialylation (siaD\(^1\)), or both capsule and LOS outer core (cps\(^3\)). Using flow cytometry, it was possible to detect strong MBL binding to the cps\(^-\) and siaD\(^-\) mutants over a wide range of concentrations. In contrast, minimal or no MBL binding was detected on the parent organism, with binding to siaD\(^-\) only at higher MBL concentrations. C4 was activated and bound by mutants that had previously bound MBL/MAST, but there was no significant difference in the amounts of C4A and C4B bound. When sialic acid residues were removed from the parent organism by neuraminidase treatment, the binding of both MBL and C4 increased significantly. Our results suggest that MBL may bind to and activate complement on these encapsulated organisms, and the major determinants of these effects are the LOS structure and sialylation.

been able to detect major differences in the MBL binding capacity of organisms that had either a normal or abnormal LOS outer core, while encapsulation appeared to have only a minor role in protecting the organism from MBL binding. We were able to correlate MBL binding with activation of the two major isomers of C4 (C4A and C4B), suggesting that the lectin can provide functional immune recognition of those organisms. These findings provide evidence that MBL and the innate immune system may have an important role in host immune responses to \textit{N. meningitidis}, particularly at stages in the life cycle of the organism when it may be nonsialylated.

### Materials and Methods

#### Preparation of MBL

MBL was prepared from cold ethanol-fractionated human plasma paste (fraction B+1 equivalent to Cohn fraction I-III, donated by Dr. C. Dash, Blood Products Laboratory, Elstree, U.K.). Ten grams of paste were dissolved in 40 ml of double-distilled water at 4°C. The resuspended paste was filtered progressively (0.22-µm pore size filter) before passage down an affinity column (10 x 15 mm) of anti-MBL (clone 131–1, Cymbus Bioscience, Southampton, U.K.) coupled to cyanogen bromide-activated Sepharose CL4B (Pharmacia Biotech, Uppsala, Sweden). The Ab column was washed extensively with PBS before elution of the MBL with 0.1 M glycine-HCl, pH 2.2. The eluent was immediately neutralized to pH 7 with 1 M NaOH, and traces of residual IgG were removed by passage through a fast protein liquid chromatography protein G column (10 x 20 mm; Pharmacia Biotech) equilibrated in 20 mM phosphate buffer.

The MBL preparation was concentrated using a Centriprep-10 (Amicon, Stonehouse, U.K.) into veronal-buffered saline supplemented with 5 mM CaCl\(_2\) and 5 mM MgCl\(_2\) (VBS 2; Stonehouse, U.K.).

#### Preparation of C4

C4A and C4B were prepared from the plasma of two donors deficient in the C4B and C4A isotypes, respectively, by chromatography on Q Sepharose FF, followed by affinity chromatography using monoclonal anti-C4 Sepharose (clone L003) and anion exchange using MonoQ (Pharmacia) (31).

#### Bacterial strains

The parent organism, \textit{N. meningitidis} B1940, and three mutants derived from it have previously been described (32). Briefly, \textit{siad} is a capsule-negative derivative caused by insertion inactivation of the polysialyltransferase gene; \textit{cpsD} is a deletion mutant in which \textit{galE} is inactive, possessing a truncated LOS lacking the outer core that cannot be sialylated; \textit{cps} lacks both a capsule and the outer LOS core caused by deletion of the entire \textit{cps} region, which includes \textit{galE} (Fig. 1). All these organisms are pilated and express the class V outer membrane proteins, Opa and Opc.

#### Growth and preparation of \textit{N. meningitidis}

The strains were removed from liquid nitrogen and cultured overnight at 37°C. Immediately before each experiment organisms were fixed by the addition of 100 µl of 2% paraformaldehyde for 10 min on ice before MBL incubation. Fixation did not disturb the binding of either MBL or C4 (described in the next section).

#### Binding of MBL to \textit{N. meningitidis}

A 50-µl aliquot of organism suspension was spun at 9470 x g for 1 min. The supernatant was removed, and the pellet was resuspended in 25 µl of VBS\(^{2+}\) containing MBL. Suspensions were incubated at 37°C for 30 min before resuspension in 25 µl of VBS\(^{2+}\) containing 10 µg/ml FITC-conjugated anti-MBL (FITC clone 131-1 manufactured by Cymbus Bioscience). The mixture of MBL and organisms was incubated at 37°C for 30 min before spinning at 9470 x g for 1 min. The supernatant was removed, and the pellet was washed with 0.5 ml VBS\(^{2+}\) before resuspension in 25 µl of VBS\(^{2+}\) containing 10 µg/ml FITC-conjugated anti-MBL (FITC clone 131-1 manufactured by Cymbus Bioscience). The mixture of MBL and
organisms were also incubated with 50 µl of a serum pool before staining for IgG, IgM, or C1q. All solutions were filtered sterilized to remove dust.

The calcium dependence and sugar specificity of binding were investigated by incubating the organisms and MBL preparation in the presence of 10 to 100 mM EDTA, galactose, or N-acetyl-glucosamine.

**Binding of C4 to N. meningitidis**

Organisms (50 µl) precoated with MBL were spun at 9470 × g for 1 min. The supernatant was removed, and the pellet was washed with 0.5 ml of VBS2+ before resuspension in 25 µl of VBS2+ containing C4 (140 µg/ml) and incubated at 37°C for 15 min. Organisms were then pelleted and washed before resuspension in 25 µl of VBS2+ containing 10 µg/ml FITC-conjugated anti-C4 (Dako Ltd., High Wycombe, U.K.) and incubation for 30 min at 37°C. Suspensions were spun at 9470 × g for 1 min. The supernatant was removed, and the pellet was washed with 0.5 ml of VBS2+ and resuspended in 100 µl of PBS. The samples were then fixed and analyzed by flow cytometry as before.

Supernatants were removed from pelleted organisms after incubation with C4, immediately mixed with an equal volume of 2× sample buffer (120 mM Tris-HCl (pH 6.75), 20% (v/v) glycerol, 4% (w/v) SDS, and 4% (v/v) 2-ME), and then stored on ice. Samples were electrophoresed using a denaturing 4 to 15% polyacrylamide gradient-SDS gel (Phast System, Pharmacia Biotech) and silver stained using a Bio-Rad silver stain kit (Bio-Rad, Hemel Hempstead, U.K.).

To confirm the absence of other activators of the classical pathway, organisms were incubated in purified human IgM (Calbiochem, San Diego, CA) at concentrations of 0.01, 0.1, and 1 mg/ml before staining for MBL and C4 binding.

**Neuraminidase treatment of the parent organism**

Organisms (50 µl) were pelleted and resuspended in 25 µl of 0.01 M K2PO4/0.025 M KCl at pH 6.0 supplemented with 10 U/ml neuraminidase (type X, Sigma) and incubated at 37°C for 4 h. Treated organisms were then incubated with the MBL preparation and C4A or C4B as described above. The success of the neuraminidase treatment was verified by incubating the organisms with a 1/50 dilution of Ab 3F11, a gift from M. Apicella (State University of New York, Buffalo, NY), for 30 min and then with a 1/50 dilution of FITC-conjugated anti-mouse IgM (Sigma) for 30 min. The Ab 3F11 recognizes the lacto-N-tetraose of the meningococcal LOS only after removal of sialic acid (33, 34).

**Statistical analyses**

Kruskal-Wallis H tests were used to determine the significance of differences in MBL and C4 binding. Differences in C4A and C4B binding were tested by calculating the difference between C4A and C4B binding in successive experiments and comparing the results to those for an organism that had not bound MBL (control) using the Mann-Whitney U test. The difference in 3F11, MBL, and C4 binding before and after neuraminidase treatment was verified by paired t tests.

**Results**

**Preparation of MBL**

MBL was present in the resuspended plasma paste at a concentration of approximately 800 ng/ml, although the presence of insoluble protein may have affected the accuracy of this quantitation. Removal was 80% complete using the anti-MBL column, and IgG removal was complete using the protein G column as shown in Figure 2. The usual recovery of MBL was 20%, with values ranging between 15 and 25% of the starting material.

A series of increasing m.w. bands was observed in the purified MBL preparation after silver staining that was identified as MBL using immunoblotting. This series of oligomeric forms was similar to that observed previously in normal serum (30), although there was a loss of the lowest molecular mass band between the 97 and 220 kDa markers after protein G chromatography. A similar loss has been observed in this laboratory using mannan column chromatography (data not shown), and the size of the band may indicate a loss of monomeric MBL.

**Binding of MBL to N. meningitidis**

Using flow cytometry it was possible to detect marked differences in the binding of MBL to the four organisms studied. Typical profiles are shown in Figure 3. In British Caucasians lacking MBL mutations, the protein is present at a median concentration of 1630 ng/ml with a 5 to 95 percentile range of 64 to 4470 ng/ml. At a concentration close to the median (1640 ng/ml), there was strong binding to the cpsD− and the cps− organisms. There was minimal or no binding to the parent organism, but there was a consistent low level of binding to the siaD− organism when the results were expressed as the percentage of organisms positive for lectin binding (p < 0.05, by Kruskal-Wallis H test). These results showed that MBL binding was possible in the presence of an intact capsule and was influenced by LOS structure.

To determine the concentration dependence of binding, we incubated organisms with concentrations of MBL ranging from 0 to 3280 ng/ml (see Fig. 4, showing mean median fluorescence). It was still possible to detect strong binding of MBL to the cps− and cpsD− mutants at physiologically low levels (410 ng/ml) of MBL. For both these organisms the median fluorescence of staining increased with increasing MBL concentration and did not reach a...
plateau. It was not possible to detect any binding of MBL to the parent organism at any of the concentrations used. A low level of binding to the siaD\textsuperscript{2} mutant was observed at 1640 and 3280 ng/ml MBL, with 1.5% of the organisms staining positive for MBL (an increase in median fluorescence of 21%) at 3280 ng/ml.

The specificity of MBL binding was investigated using various carbohydrate ligands for MBL. Binding of MBL was completely inhibited by N\textsuperscript{-}acetyl-glucosamine at a concentration of 25 mM, whereas galactose at this concentration had no effect. This profile of sugar-specific competition is consistent with the known sugar binding profile of MBL (1). The addition of 10 mM EDTA to the MBL preparation also completely inhibited binding, which is consistent with the known calcium-dependent nature of MBL binding (data not shown).

**Binding of C4**

To further investigate the biologic effector functions of MBL binding, we evaluated C4 deposition on the surface of the organisms after incubation with the MBL preparation. We were also able to compare the binding of the two major isotypes of C4, C4A and C4B (see Fig. 5).

When organisms were incubated with MBL at 3280 ng/ml and then exposed to C4 (140 μg/ml), high levels of C4 binding were observed with the cps\textsuperscript{2} and cpsD\textsuperscript{2} organisms compared with organisms that had not been incubated with MBL (p < 0.05, by Kruskal-Wallis H test), i.e., those organisms that had bound MBL strongly. In contrast, no C4 was bound by the parent organism above the background. A low level of C4 was bound by the siaD\textsuperscript{2} organism, reflecting the low uptake of MBL on this organism. There was no statistically significant difference in the binding of C4A and C4B to the cps\textsuperscript{2} and cpsD\textsuperscript{2} organisms compared with the binding of these isoforms to the parent organism (by Mann-Whitney U test). These data are summarized in Figure 6.

It was not possible to detect any IgG or C1q bound to the organisms before or after incubation of the organisms with the MBL preparation. It was possible to detect very low levels of IgM binding to the cps\textsuperscript{2} and cpsD\textsuperscript{2} organisms after incubation with the MBL preparation. However, when pooled serum was incubated with the organisms, much higher levels of binding of IgG, IgM, and C1q were observed in each case (data not shown). To confirm the absence of classical pathway activators in the MBL preparation, the organisms were incubated with purified IgM at concentrations ranging from 0.01 to 1 mg/ml before incubation with MBL and C4. There was no increase in MBL or C4 binding with increasing IgM concentration (data not shown).

Since most of the C4b generated by classical pathway activation does not bind to nearby surfaces, we postulated that it might be possible to detect this product in the supernatant of organisms that

**FIGURE 3.** Representative flow cytometric profiles of MBL binding to the parent and three mutants of *N. meningitidis* using MBL at a final concentration of 3280 ng/ml. The control histogram (shaded) shows samples incubated with FITC-conjugated anti-MBL alone, with the experimental samples (thick line) in which samples have been incubated with MBL and then FITC-conjugated anti-MBL. This experiment was repeated four times.

**FIGURE 4.** MBL binding to *N. meningitidis* B1940 and three isogenic mutants at different lectin concentrations. Binding of MBL has been expressed as the median fluorescence. Each point represents the mean of four experiments, with error bars indicating the SEM.

**FIGURE 5.** Representative flow cytometric profiles of C4A binding to the parent and two mutants of *N. meningitidis* using MBL at 3280 ng/ml in each case. The control histogram (shaded) shows C4 binding to each mutant without prior incubation with MBL. The thick line shows C4 binding after incubation with MBL. Each mutant was incubated with 140 μg/ml C4.

**FIGURE 6.** C4A and C4B binding to *N. meningitidis* B1940 and three isogenic mutants after incubation with MBL at 3280 ng/ml. Results have been summarized as the median fluorescence, with each data point representing the mean of five or six experiments ± SEM. Each mutant had been incubated with 140 μg/ml C4 after incubation with MBL.
had bound C4. In C4 activation, the 93-kDa C4 \( a \)-chain is cleaved to give the 83-kDa C4 \( a \)9-chain, and we sought evidence for the generation of this in supernatants of organisms that had bound C4.

As shown in Figure 7, it was possible to demonstrate the cleavage of the C4 \( a \)-chain to the \( a \)9-chain with the \( cps^* \) and \( cpsD^* \) organisms that bind C4, but not in the supernatants of the parent or \( siaD^* \) organisms that bind little or no C4. No cleavage of C4 was detected in the absence of MBL (bottom panel). M, molecular mass markers in kilodaltons.

Neuraminidase treatment of the parent organism

The results of the above experiments suggested that LOS structure is an important determinant of MBL binding. The two mutants that bound MBL have mutations that result in a truncated LOS. However, they differ from most pathologic strains of \( N. \) meningitidis in that they lack the terminal lactoneotetraose, which can be endogenously and exogenously sialylated in vivo. Sialylation of LOS is an important virulence factor that influences bacterial adhesion and survival in human hosts. We therefore investigated the effect of sialic acid removal from the parent organism to determine the role of sialylation in MBL binding.

After neuraminidase treatment, a significant increase in MBL binding was detected compared with that in organisms incubated in buffer alone (Fig. 8). Similarly, neuraminidase treatment was shown to increase binding of mAb 3F11, which recognizes a terminal component of the LOS, but only if the epitope has not been obscured by the addition of sialic acid (33, 34). It was of note that the entire population of organisms did not stain positive for MBL and 3F11. This binding of 3F11 to only a subset of the population is similar to previous reports using an ELISA format for 3F11 detection (35, 36), which, unlike flow cytometry, has the advantage of enzyme amplification in the detection stage. The removal of sialic acid was also accompanied by a rise in C4A and C4B deposition on the organism.

While only a proportion of the organisms stained positive for C4 binding (16% for C4A, 12% for C4B), the intensity of C4 staining in the positive population was comparable to the staining observed previously in the \( cps^* \) and \( cpsD^* \) organisms. Similar proportions of the population were stained for 3F11 and MBL, while the different absolute values presumably indicate either a difference in detection sensitivity or a difference in the binding sites for 3F11 and MBL on the desialylated organism.

Discussion

MBL is a constitutively expressed immune protein. Deficiency of the protein has been associated with recurrent infections, but the
role of the protein in normal host defense has yet to be defined. MBL binds to a range of carbohydrates, activates the classical complement pathway in an Ab-independent manner, and is believed to act directly as an opsonin (25). Although the binding to monosaccharides has been well defined (1), the binding to complex saccharides, in particular to microbial structures, is poorly understood. However, one recent study has defined the carbohydrate groups to which MBL can bind in complex saccharides presented at sufficient density in *M. avium* (27).

In the present study we have used flow cytometry to investigate the interaction of MBL with a strain of the organism *N. meningitidis* serogroup B (B1940) and three isogenic mutants. The four organisms studied fell clearly into two groups. Using the collectin at a concentration (1640 ng/ml) close to the median value of the British population (12), the organisms with intact LOS (parent and siaD−) did not bind MBL or bound MBL only weakly, whereas the organisms with the truncated LOS (cps− and cpsD−) bound MBL strongly. Interestingly, both cps− and cpsD− bound MBL strongly even at levels of MBL (410 ng/ml) close to the median value for an individual heterozygous for an MBL structural mutation (358 ng/ml for British Caucasians) (12). Thus, the responses of normal and heterozygous individuals could theoretically be broadly similar when these two organisms are targeted by the MBL/MASP system.

Our data suggest that it is the LOS structure and not encapsulation that is the major determinant of MBL binding. Previously, van Emmerik et al. (26) reported that encapsulation of *N. meningitidis* significantly inhibited the binding of MBL compared with that in unencapsulated strains. However, we observed only small, but reproducible, differences in MBL binding to WT and siaD− organisms at high concentrations of the lectin, suggesting that the capsule may afford some protection to the organism in a host with a high serum MBL level.

This study has permitted us to address several aspects of MBL-initiated complement activation by neisserial organisms. In our study no significant differences were observed between the binding of C4 to the cps− or cpsD− mutants. The lower C4 binding to the siaD− mutant was presumably a reflection of the lower MBL binding capacity of this organism. These observations on C4b binding parallel recent studies of C3b deposition using these same mutants (56). All three of the mutants were shown to be serum susceptible, but C3b deposition via the classical pathway occurred predominantly on the cps− and cpsD− mutants regardless of the presence or the absence of the capsule. In these experiments enhanced complement activation correlated to IgM binding on these mutants, suggesting that the complement activation was at least partly Ab mediated.

We were unable to detect significant differences in the binding of C4A and C4B to the two organisms that bound C4. The reaction mechanism by which the C4b fragments of C4 make covalent bonds with nearby surfaces is now understood in some detail, and the evidence indicates that the C4b isotype may be the more important (37). It had been suggested that C4b deficiency might predispose to bacterial meningitis (38) and be a risk factor for bacteremia with several encapsulated organisms (39). However, using a larger series, Cates et al. (40) were unable to confirm any increase in C4b deficiency among patients with bacteremia or meningitis caused by encapsulated bacteria. Our data appear to support this study, since C4a has the same capacity to bind to the two different mutants as C4B.

We have considered the possibility that C4 activation might occur by a mechanism other than MBL-MASP. Using pooled normal sera, IgG and IgM will bind to all the mutants, allowing C1q to bind and presumably activate the classical pathway. However, the absence of C1q and IgG on the surface of organisms preincubated with the MBL preparation supports our interpretation that the activation and cleavage of C4 must only have occurred through the MBL-MASP pathway. It was possible to detect very low levels of IgM on the surface of the cps− mutant, presumably reflecting trace contamination of the MBL preparation. Although IgM has been reported to act as a ligand for MBL (41), we were unable to increase MBL binding by incubating the organisms with human IgM before MBL incubation. The IgM binding we detected is therefore unlikely to account for the MBL binding observed on the mutants. We were also unable to increase C4 activation after prior incubation with increasing IgM concentrations, which further confirms the absence of C1q in the MBL preparation.

Serogroup B meningococci are responsible for most meningococcal infections in Northern Europe and the U.S. It is thought that meningococcal infection results from colonization of the nasopharynx followed by invasion of the respiratory epithelium and entry into the circulation. Once in the blood, organisms can proliferate in susceptible individuals, causing either sepsis, with which most mortality is associated, or focal infections such as meningitis (42). It is not clear why some patients develop sepsis, whereas others resist infection or are only mildly affected, but a number of host and bacterial factors have been implicated.

The majority of meningococci isolated from patients with invasive disease are encapsulated. The polyneuraminic acid capsule of the organism is therefore an important virulence factor and acts by resisting complement lysis and phagocytosis (43) by down-regulation of the alternative pathway. Furthermore, the capsule is only weakly immunogenic due to mimicry of the human neural cell adhesion molecule (44).

More recently, it has been discovered that sialylation of the LOS is also an important virulence factor. The majority (>-97%) of organisms isolated from blood and CSF have LOS structures (immunotypes) that can be sialylated (45). In contrast, the LOS immunotypes of carriers are heterogeneous, with a minority capable of LOS sialylation. The significance of LOS sialylation has been further demonstrated in an infant rat model (46), where it was found to be almost as important as capsulation in protecting the organism from host attack. Similar results have been demonstrated in a human whole blood model of meningococcal bacteremia (47). It would appear that LOS sialylation is necessary to confer complete serum resistance to this organism (32).

In the present study the lack of the sialic acid acceptor site appeared to favor MBL binding and complement activation. We therefore attempted to remove the sialic acid from the LOS of the parent organism using neuraminidase. After removal of some of the sialic acid from the LOS, MBL was able to bind and, through MASP activation, promote deposition of C4A and C4B on the surface of the organism. Interestingly, the median fluorescence of organisms that had bound C4 was similar to that seen with the mutant strains, indicating that, in a nonsialylated state, the parent organism may be as susceptible to MBL attack as the truncated LOS mutants.

LOS sialylation may not just be of importance in protecting organisms from host defenses, since it has been shown to influence adherence to epithelial and vascular surfaces (47). The attachment of meningococci to these cells is necessary for invasion of the nasopharyngeal epithelium and migration out of the vascular system to other tissues. The report of a patient with meningococcal orbital cellulitis in which isolates were obtained from both blood and periorbital aspirates (48) confirms the capacity of *N. meningitidis* to modulate LOS sialylation during infection. In addition to influencing bacterial traffic and survival, LOS sialylation plays a
role in determining host inflammatory responses, a critical factor in meningococcal disease (49, 50).

There are two probable mechanisms by which MBL could influence host defense against N. meningitidis; firstly by MBL-medi- ated bacterial killing and secondly by inflammatory modulation. In the presence of a replete complement system, the MBL-MASP system should lead to the generation of terminal attack complexes and bacterial lysis. Other studies have also demonstrated that MBL can enhance opsonophagocytosis, even in the absence of serum and bacterial lysis. Other studies have also demonstrated that MBL

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