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Regulation of the Mannan-Binding Lectin Pathway of Complement on *Neisseria gonorrhoeae* by C1-Inhibitor and \(\alpha_2\)-Macroglobulin

Sunita Gulati,* Kedarnath Sastry, † Jens C. Jensenius, ‡ Peter A. Rice,* and Sanjay Ram*

We examined complement activation by *Neisseria gonorrhoeae* via the mannan-binding lectin (MBL) pathway in normal human serum. Maximal binding of MBL complexed with MBL-associated serine proteases (MASPs) to *N. gonorrhoeae* was achieved at a concentration of 0.3 \(\mu\)g/ml. Preopsonization with MBL-MASP at concentrations as low as 0.03 \(\mu\)g/ml resulted in \(~60\%\) killing of otherwise fully serum-resistant gonococci. However, MBL-depleted serum (MBLdS) reconstituted with MBL-MASP before incubation with organisms (postopsonization) failed to kill at a 100-fold higher concentration. Preopsonized organisms showed a 1.5-fold increase in C4, a 2.5-fold increase in C3b, and an \(~25\)-fold increase in factor Bb binding; enhanced C3b and factor Bb binding was classical pathway dependent. Preopsonization of bacteria with a mixture of pure C1-inhibitor and/or \(\alpha_2\)-macroglobulin added together with MBL-MASP, all at physiologic concentrations before adding MBLdS, totally reversed killing in 10% reconstituted serum. Reconstitution of MBLdS with supraphysiologic (24 \(\mu\)g/ml) concentrations of MBL-MASP partially overcame the effects of inhibitors (57% killing in 10% reconstituted serum). We also examined the effect of sialylation of gonococcal lipooligosaccharide (LOS) on MBL function. Partial sialylation of LOS did not decrease MBL or C4 binding but did decrease C3b binding by 50% and resulted in 80% survival in 10% serum (lacking bacteria-specific Abs) even when sialylated organisms were preopsonized with MBL. Full sialylation of LOS abolished MBL, C4, and C3b binding, resulting in \(100\%\) survival. Our studies indicate that MBL does not participate in complement activation on *N. gonorrhoeae* in the presence of “complete” serum that contains C1-inhibitor and \(\alpha_2\)-macroglobulin. *The Journal of Immunology*, 2002, 168: 4078–4086.

Mannan-binding lectin (MBL) is a member of the collectin family of proteins (1–3), found in the serum of all mammals and birds that have been studied. MBL binds to a wide array of carbohydrate structures on microbial surfaces and is believed to mediate direct killing via complement activation (4–9) or by enhancing phagocytosis by acting as an opsonin (10, 11). Recently, complement receptor 1 (CR1/CD35) has been defined as the cellular receptor for MBL (12, 13), a finding consistent with the structural relatedness of MBL to C1q, which also binds to CR1 (14, 15). MBL is associated with three novel MBL-associated serine proteases (MASPs), MASP-1 (16, 17), MASP-2 (18), and MASP-3 (19). MASP-1 appears to activate C3 directly (18, 20, 21), while MASP-2 (21, 22) has C4-cleaving activity. Although the function of MASP-3 is not resolved, recombinant MASP-3 can inhibit C4-cleaving activity of recombinant as well as naturally occurring MBL-MASP-2 complexes (19).

MBL preferentially recognizes glucans, lipopolysaccharides, and glycoconjugate phospholipids that contain mannose, glucose, fucose, or \(N\)-acylglycosamine (GlcNAc) as their terminal hexose (Hex) (23). The three-dimensional structure of trimeric human MBL-carbohydrate recognition domains shows that ligands must span 45–50 Å between the binding sites to achieve high-affinity binding (23, 24). Thus MBL is efficient in recognizing microbial surfaces with a high content of repetitive (and terminal) mannose and/or GlcNAc residues, such as those presented by *Saccharomyces cerevisiae* (10), *Candida albicans* (25), *Escherichia coli* strain K12 (5, 26–28), *Salmonella typhimurium* (5, 28–31), *Salmonella montevideo* (5, 28–31), gp120 of HIV-1 (32, 33), gp110 of HIV-2 (32, 33), and *Neisseria gonorrhoeae* (29). Such carbohydrate micropatterns are found in limited amounts in glycoproteins of higher animals, and these are not arranged in a repetitive pattern in the membrane that would be suitable for binding to MBL (34). Furthermore, mammalian carbohydrates often terminate in sialic acid residues, which shield the relevant neutral sugars, and thus are not recognized by MBL (34). This finding may also extend to prokaryotes where, for example, sialylation of the lipooligosaccharide (LOS) of *N. gonorrhoeae* also decreases MBL binding (29).

Prior studies of MBL binding and function on microbial surfaces have been conducted using purified MBL to preopsonize bacteria, followed by the addition of pure C3 or C4 (5, 26, 31, 32, 35). However, such a system may not always reflect in vivo circumstances, where microbes likely encounter MBL together with other serum components, including two known inhibitors of the MBL pathway, C1-inhibitor (C1-INH) and \(\alpha_2\)-macroglobulin (\(\alpha_2\)M) (36–40). \(\alpha_2\)M binds to MASP-1 covalently and to MBL in a noncovalent \(\mathrm{Ca}^{2+}\)-dependent manner to inhibit complement activation by the MBL-MASP complex (39). C1-INH forms stable

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3 Abbreviations used in this paper: MBL, mannan-binding lectin; MASP, MBL-associated serine protease; NANA, \(N\)-acyetylneuraminic acid; \(\alpha_2\)M, \(\alpha_2\)-macroglobulin; LOS, lipooligosaccharide; Hep, heptose; NHS, normal human serum; C1-INH, C1-inhibitor; GlcNAc, \(N\)-acylglycosamine; CR1, complement receptor 1; C4bp, C4b-binding protein; Hex, hexose.
equimolar complexes with both MASP-1 and MASP-2 and inhibits their proteolytic activities (41). In addition, C3-convertases generated by MBL-MAST on erythrocytes are exquisitely susceptible to regulation by C4b-binding protein (C4bp) and factor H; this regulation may occur because MBL enhances binding of C4bp to C4b and factor H to C3b (8). Relevant to the current study, these observations suggest that, like other pathways of complement that are tightly regulated on the gonococcal surface (42), activation by MBL may also be regulated.

The present study was undertaken initially to define the complement-activating role of MBL on the gonococcal surface in what we believed to be a more likely simulation of a physiologic circumstance (i.e., an environment where the microbe encounters MBL together with other serum components). We demonstrate that MBL indeed activates complement, causing bacterial killing, but only when organisms are preopsonized with pure MBL-MAST (consistent with prior reports), and not when MBL-MAST complexes are added to bacteria concomitantly with serum (postopsonization). In addition, we examined the roles of C1-INH and αM in inhibiting MBL-induced complement activation on the bacterial surface as a possible explanation for the lack of activity of MBL when used together with these serum proteins. We also examined the effect of sialylation of gonococcal LOS on the ability of MBL to activate complement and kill N. gonorrhoeae.

Materials and Methods

Bacterial strains and growth methods

MBL-bacterial interactions were examined using N. gonorrhoeae strains 1291a and 24-1. The LOS structures of each of these strains has been characterized previously (45, 46), and the glyco substitutions of core heptose (Hept) are indicated in Table I. 1291a expresses a single LOS species, its Hept chain terminating in a GlcNAc residue (45, 47). Gonococcal LOS species that sialylate possess a terminal Galβ1→4GlcNAc (lactosamine) residue (48). Therefore, the LOS of strain 1291a cannot be sialylated. In contrast, 24-1 expresses one major and two minor glycoforms (Table I); glycoform(s) that terminate in a Hexβ1→4HexNAc residue (presumed to be terminal lactosamine) can be sialylated (46). Gonococcal strains were inoculated onto chocolate agar plates supplemented with the gonococcal liquid medium (49). Sialylation of 24-1 LOS was performed by adding human C2 were purchased from Advanced Research Technologies (San Diego, CA). Biologically active C2 was dispensed into each microtiter well. Microtiter wells were incubated for 3 h at 37°C on a horizontal shaker at 200 Hz to allow capture of bacteria, followed by three washes with PBS-0.05% Tween 20. Primary and secondary Abs were used at the concentrations indicated above and were allowed to react at 37°C for 1 h. After washing, p-nitrophenyl phosphate substrate was added, followed by incubation at 25°C. Plates were read 10 min after adding substrate.

Bactericidal assay

Serum bactericidal assays were performed as described previously (51). Briefly, 2000 CFU/ml N. gonorrhoeae grown to mid-log phase were incubated with indicated concentrations of serum in a final volume of 150 μl. Duplicate samples were plated at 0 and 30 min; survival was expressed as a percentage of CFU at 30 min divided by CFU at 0 min. Bacterial growth that was sometimes observed after 30 min (i.e., survival >100%) was assigned a value of 100%. In some assays, bacteria were preincubated with pure MBL-MAST (with or without C1-INH) and/or αM, amount specified below for each experiment) for 15 min at 37°C before the addition of serum. Final volumes of all reaction mixtures were maintained at 150 μl.

Flow cytometry

Binding of pure factor H to the bacterial surface was quantified by flow cytometry (FACSscan; BD Immunocytometry Systems, San Jose, CA), as described previously (42, 43). Briefly, 10^7 bacteria in 2 M were incubated with 5 μg pure human factor H in a final volume of 100 μl for 30 min at 37°C. Bacteria were then washed once, and factor H was detected using affinity-purified goat anti-human factor H (see above), followed by anti-goat IgG-FITC at a dilution of 1/50.

Results

MBL binding to N. gonorrhoeae strains 1291a and 24-1 is dose dependent and saturable.

We initially screened 20 strains of N. gonorrhoeae (9 sensitive and 11 resistant to the bactericidal action of 10% normal human serum

Antibodies

mAb to MBL was kindly provided by Dr. R. A. B. Ezekowitz (Harvard Medical School, Boston, MA) (50). mAbs C-5G and G-3E, which are specific for C3b and iC3b, respectively (55), were kindly provided by Dr. K. Iida (Takeda Chemical Industries, Tsukuba, Japan). C4 bound to organisms was quantified either using mAb against the C4d fragment (Qiudel, San Diego, CA) or goat polyclonal anti-human C4 (The Scripps Laboratory, La Jolla, CA). The anti-C4d mAb was used in experiments with 1291a, because C4bp binds to this strain (our unpublished observations), which could result in processing of C4b deposited on the organism to the ~145-kDa C4c fragment (released into solution) and the smaller ~45-kDa C4d fragment that remains bound to the organism. Therefore, quantifying C4d binding more accurately reflects the total number of C4 molecules deposited on the organism and is not influenced by additional processing by C4bp and factor I. Surface-bound factor Bb was detected using an anti-factor Bb mAb (Quiadel). All mAbs were used at a concentration of 1 μg/ml in whole-cell ELISA. Alkaline phosphatase-conjugated anti-mouse IgG or anti-goat IgG (Sigma-Aldrich) were used to detect to use these components bound to bacteria in whole-cell ELISA (see below). Cell culture supernatant of mAb 2C3 (containing ~25 μg/ml specific Ab), which is specific for the H.8 gonococcal Ag (56), was used as a detector to ensure uniform capture of bacteria affixed to microtiter wells in whole-cell ELISA (57). Alkaline phosphatase-conjugated anti-mouse IgG or anti-goat IgG (Sigma-Aldrich) was used as a secondary or disclosing Ab. All polyclonal Abs and conjugates were diluted 1/1000 in PBS-0.05% Tween 20. Factor H that bound to bacterial surfaces was detected by flow cytometry using affinity-purified goat anti-human factor H at a concentration of 10 μg/ml (kind gift of Dr. M. K. Pangburn, University of Texas Health Sciences Center, Tyler, TX), and disclosed using FITC-conjugated anti-goat IgG (Sigma-Aldrich) at a dilution of 1/50.

ELISA

Whole-cell ELISA was used to detect MBL, Ig, and complement components that bound to bacteria, as described previously (57). Briefly, 5 × 10^5 bacteria suspended in HBSS containing 0.15 mM CaCl_2 and 1.1 mM MgCl_2 (HBSSC) were incubated with serum or pure MBL-MAST at 37°C (concentration and incubation time are specified below for each experiment). Suspensions were then centrifuged at 5000 × g for 20 min at 4°C and pellets were washed twice with cold HBSS containing 5 mM PMSF. Pellets were then resuspended in 200 μl of HBSS containing PMSF, and 50 μl was dispensed into each microtiter well. Microtiter wells were incubated for 3 h at 37°C on a horizontal shaker at 200 Hz to allow capture of bacteria, followed by three washes with PBS-0.05% Tween 20. Primary and secondary Abs were used at the concentrations indicated above and were allowed to react at 37°C for 1 h. After washing, p-nitrophenyl phosphate substrate was added, followed by incubation at 25°C. Plates were read 10 min after adding substrate.

Serum obtained fresh from 11 normal adults who had no history of neisserial infection were pooled and stored at ~80°C until used. MBL-deficient serum was obtained from an individual who had no demonstrable MBL by ELISA (50). In some experiments, serum was absorbed against glutardehyde-fixed N. gonorrhoeae strain 24-1 for 1 h on ice to deplete serum of Abs against this strain (51). All sera (including absorbed sera) contained normal classical and terminal hemolytic activity as determined by the Total Hemolytic Complement kit (The Binding Site, Birmingham, U.K.). MBL-MAST complexes, containing both MASP-1 and MASP-2, were obtained as a sterile solution stabilized with 0.5% (w/v) human serum albumin from Statens Serum Institut (Copenhagen, Denmark) and further purified using carbohydrate affinity chromatography followed by ion exchange and gel permeation chromatography, as described previously (patent no. W099/64453, Ref. 52). Biological activity of the purified MBL-MAST was confirmed by previously described methods (53). Purified human factor H and C1-INH were purchased from Sigma-Aldrich (St. Louis, MO). We also used a second source of αM that was the kind gift of L. Sottrup-Jensen (University of Aarhus, Denmark) (54) to avoid concern that trehalose present in the buffer of the commercially available αM preparation might account for MBL-inhibitory function (36, 39, 40). C2-depleted serum and purified human C2 were purchased from Advanced Research Technologies.

Sera and complement reagents

Serum Abs against this strain (51). All sera (including absorbed sera) contained normal classical and terminal hemolytic activity as determined by the Total Hemolytic Complement kit (The Binding Site, Birmingham, U.K.). MBL-MAST complexes, containing both MASP-1 and MASP-2, were obtained as a sterile solution stabilized with 0.5% (w/v) human serum albumin from Statens Serum Institut (Copenhagen, Denmark) and further purified using carbohydrate affinity chromatography followed by ion exchange and gel permeation chromatography, as described previously (patent no. W099/64453, Ref. 52). Biological activity of the purified MBL-MAST was confirmed by previously described methods (53). Purified human factor H and C1-INH were purchased from Sigma-Aldrich (St. Louis, MO). We also used a second source of αM that was the kind gift of L. Sottrup-Jensen (University of Aarhus, Denmark) (54) to avoid concern that trehalose present in the buffer of the commercially available αM preparation might account for MBL-inhibitory function (36, 39, 40). C2-depleted serum and purified human C2 were purchased from Advanced Research Technologies.
(NHS)) for binding to pure MBL-MASP by whole-cell ELISA and compared binding between the two groups to assess a possible correlation between MBL-MASP binding and a serum-sensitive phenotype. The mean ± SE MBL-MASP binding (measured as OD$_{410}$ readings at 10 min) to serum-sensitive strains was 0.404 ± 0.093, and that for serum-resistant strains was 0.292 ± 0.053. This difference was not statistically significant ($p = 0.29$, ANOVA) and suggested to us at the outset that MBL binding may not be an overriding variable in determining the sensitivity of gonococci to complement-mediated killing. In all ELISA, we detected H.8 Ag that is expressed by all pathogenic Neisseria species using mAb 2C3 (56) to ensure uniform capture of organisms onto microtiter wells. H.8 OD$_{410}$ ELISA readings at 10 min for all strains ranged from 0.6 to 0.8, ensuring uniform bacterial capture onto microtiter wells.

Among the serum-resistant strains tested, the highest binding was seen with strain 1291a, which was not surprising because the Hep1 chain of the LOS of this strain terminates in a GlcNAc residue (see Table 1), a known ligand for MBL (1, 23). Strain 24-1 showed the second highest level of binding among the serum-sensitive strains tested. Unlike 1291a, 24-1 has the ability to sialylate its LOS (57), and the effects of LOS sialylation upon MBL binding and function were studied using the sialylated derivative of 24-1 (24-1 NANA).

We examined the effect of increasing MBL concentrations on MBL binding to strains 1291a and 24-1 (Fig. 1). Maximal binding to each strain was achieved with MBL concentrations as low as 0.3 μg/ml.

### Table 1. Hexose substitutions on Hep1 chain of LOS of strains 1291a and 24-1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hexose Extensions Off Hep1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1291a</td>
<td>GlcNAc-Gal-Glc-Hep1</td>
</tr>
<tr>
<td>24-1</td>
<td>Hex-HexNAc-Hex-Hex-Hep1</td>
</tr>
</tbody>
</table>

* The identities of the hexoses have not been defined (46).

Functional correlates of MBL binding: MBL-mediated killing requires preopsonization of organisms

We studied the functional effects of MBL binding in a bactericidal assay using two different opsonizing conditions. The effects of MBL on 1291a will first be considered, and the influence of sialylation upon MBL function will be discussed later. Strain 1291a was either 1) preopsonized with pure MBL (final MBL concentrations in the reaction mixture ranging from 0.003 to 3 μg/ml in log$_{10}$ increments) followed by the addition of MBL-deficient serum (to a final concentration of 10%), or 2) added to MBL-deficient serum that had been reconstituted with MBL-MASP, using increments in concentration as indicated above (postopsonization). We observed killing of 1291a only when organisms were preopsonized with pure MBL, followed by addition of MBL-deficient serum. Preopsonization with 0.03 μg/ml MBL-MASP resulted in 57% killing; 10- and 100-fold increases in MBL-MASP concentrations resulted in 75 and 88% killing, respectively (Fig. 2A). Reconstituting MBL-deficient serum with pure MBL-MASP (postopsonization) before addition of bacteria resulted in no killing with MBL-MASP concentrations up to 3 μg/ml (dotted baseline in Fig. 2A). We also examined the effect of serum concentration on killing of strain 1291a preopsonized while holding MBL-MASP concentrations fixed (0.03 μg/ml) (Fig. 2B). We observed 65% killing of organisms at a serum concentration of 67%; killing decreased (Fig. 2B) in a dose-dependent fashion as serum concentration was decreased. As a negative control, we postopsonized bacteria with 67% MBL-deficient serum reconstituted with MBL-MASP (0.03 μg/ml) and observed no killing.

The lack of killing of 1291a by MBL-deficient serum reconstituted with pure MBL before addition of bacteria suggested inhibition of the complement-fixing ability of MBL by one or more inhibitors in serum.

Characterization of Ab and complement binding to 1291a

Using whole-cell ELISA, we examined binding of IgG, IgM, C4d, C3b, iC3b, and factor Bb binding to 1291a that was 1) preopsonized with MBL-MASP followed by addition of MBL-depleted serum, or 2) postopsonized with MBL-depleted serum that had been reconstituted with MBL-MASP (Fig. 3). In both instances, the MBL-depleted serum constituted 10% of the reaction volume in reconstituted serum, or 2) added to MBL-depleted serum that had been reconstituted with MBL-MASP (Fig. 3). In both instances, the MBL-depleted serum constituted 10% of the reaction volume. The lack of killing of 1291a by MBL-deficient serum reconstituted with pure MBL before addition of bacteria suggested inhibition of the complement-fixing ability of MBL by one or more inhibitors in serum.

![FIGURE 1. Binding of MBL-MASP to N. gonorrhoeae strains 1291a and 24-1. Bacteria (5 × 10⁷ CFU/ml) were incubated with increasing concentrations of MBL-MASP (ranging from 0.003 to 3 μg/ml in log₁₀ increments) for 30 min at 37°C. Bacteria were washed twice and dispersed into microtiter wells, followed by detection of bound MBL using anti-MBL-specific mAb. Both strains showed dose-responsive and saturable binding; maximal binding was achieved at 0.3 μg/ml. Equal capture of bacteria onto microtiter wells was monitored using mAb 2C3 that is specific for the lipoprotein H.8 Ag that is expressed equally by all N. gonorrhoeae (96). Each data point represents the mean ± SD of two separate experiments performed in duplicate.](http://www.jimmunol.org/)
Complement activation on preopsonized 1291a requires an intact classical pathway

Recruitment of C3b and factor Bb on preopsonized organisms could occur either by direct activation of C3b by MBL-MASP-1 or by activation of C3 via classical pathway C3-convertase (C4b, 2a). This was addressed by adding either C2-depleted serum or C2-depleted serum reconstituted with purified human C2 at physiologic concentrations (25 μg/ml) to 1291a preopsonized with MBL-MASP. C3b and factor Bb binding were augmented when the classical pathway was intact (Fig. 4, filled bars). Controls for this experiment included organisms that were postopsonized with C2-depleted serum reconstituted with C2 and pure MBL-MASP (data not shown), which showed a similar complement activation profile as preopsonized organisms treated with C2-depleted serum (Fig. 4, hatched bars). These data suggest that C3b and factor Bb deposition on 1291a preopsonized with MBL-MASP was not entirely the result of direct C3 activation; an intact classical pathway contributed significantly to augment C3b and factor Bb deposition.

C1-INH and α2M act synergistically to inhibit MBL function on the surface of 1291a

The previous data indicated that MBL activated complement only when organisms were preopsonized with MBL-MASP. Reconstitution of MBL-deficient serum with 10× concentrations of MBL-MASP complexes did not result in bacterial killing. C1-INH and α2M are two known regulators of the MBL pathway (37–40). We studied the effects of these two inhibitors on MBL-induced complement activation (measured by bacterial killing) on the surface of strain 1291a. Organisms were preopsonized for 15 min with either
MBL-MASP alone or a mixture of MBL-MASP, either with C1-INH or α2M, or both (Fig. 5A). The ratios of MBL-MASP to the inhibitors were based on those in NHS, which assumed concentrations of MBL-MASP, C1-INH, and α2M in NHS of 3, 200, and 2500 μg/ml, respectively (Fig. 5A, shaded bars), or in a 10-fold excess amount of MBL relative to the concentration of the inhibitors (Fig. 5A, filled bars). The MBL-MASP, alone or with inhibitors, were added to bacteria and allowed to incubate at 37°C for 15 min. This was followed by the addition of MBL-depleted serum to a final concentration of 10%, and the reaction was incubated for 30 min at 37°C. As expected, ≥97% killing was seen with organisms preopsonized with MBL alone (at both concentrations). Preopsonization with MBL-MASP plus C1-INH decreased killing to 37% when concentrations of the pure components were physiologically balanced, but it did not confer protection (96% killing) when a 10-fold relative excess of MBL-MASP was used. Greater levels of protection against MBL-MASP-mediated killing were offered by α2M alone; no killing (0%) occurred when the two pure components were physiologically balanced, and 55% killing was seen with supraphysiologic (10X) MBL-MASP concentrations. When MBL-MASP was added to 1291a in the presence of both C1-INH and α2M, no killing was observed at either MBL-MASP concentration, suggesting that these two inhibitors acted synergistically to inhibit MBL-induced bacterial killing. Similar results were obtained with both sources of α2M and C1-NH. Next, we reconstituted MBL-deficient serum with increasing amounts of MBL-MASP in an effort to overcome the inhibitory influence of endogenous C1-INH and α2M present in MBL-deficient serum (Fig. 5B). Consistent with the above observations, no killing was observed when MBL-deficient serum was reconstituted with physiologic or 10X concentrations of MBL-MASP; killing increased thereafter in a dose-responsive fashion as MBL-MASP concentrations in serum exceeded 20X (Fig. 5B).

Effects of gonococcal LOS sialylation on MBL binding and function

We used strain 24-1 and its sialylated derivative, 24-1 NANA (57), to study the influence of LOS sialylation on MBL function. Because 24-1 is a highly serum-sensitive strain, we first absorbed MBL-deficient serum using glutaraldehyde-fixed organisms (51) to deplete serum of bacterial-specific Abs. This permitted complement activation by MBL alone, in the absence of specific Abs.

Complement component binding to 24-1 grown in the presence of increasing amounts of CMP-NANA

We measured MBL, C4, C3b, iC3b, factor Bb, and factor H binding to 24-1 grown in the presence or absence of CMP-NANA. Growth medium was supplemented with CMP-NANA at concentrations of 2–100 μg/ml. Complement activation and binding on the bacterial surface was measured using whole-cell ELISA, except binding of factor H (added pure), which was assessed by flow cytometry. Three conditions of opsonization were studied: 1) sia
ylated organisms preopsonized with pure MBL-MASP, followed by the addition of absorbed MBL-deficient serum; 2) MBL-deficient serum (absorbed) reconstituted with MBL-MASP added to sialylated bacteria (postopsonization); and 3) as a positive control, sialylated organisms (24-1 NANA, not preopsonized) incubated with MBL-deficient serum (not absorbed) having intact Ab-dependent classical and alternative pathways. Results of this experiment are shown in Fig. 5. Postopsonization resulted in no C4, C3b, iC3b,
or factor Bb binding to 24-1 or any of its sialylated counterparts (Fig. 6). Complement activation proceeded unimpeded on 24-1 when the Ab-dependent classical and alternative pathways were intact, and binding of C4, C3b, and factor Bb decreased as the concentration of CMP-NANA in growth medium was increased. Binding of factor H and the resultant cleavage product iC3b reached a maximum when only 2 μg/ml CMP-NANA was used in growth medium. MBL binding to 24-1 was similar to that seen with 24-1 NANA at this same concentration of CMP-NANA in growth medium and decreased thereafter in a dose-responsive fashion with increasing CMP-NANA concentrations. C4 binding to bacteria paralleled MBL binding when organisms were preopsonized with MBL-MASP (Fig. 6, filled bars). Despite similar C4 binding to 24-1 (unsialylated) and 24-1 NANA (2 μg/ml CMP-NANA), C3b binding to 24-1 NANA decreased by >50% compared with 24-1 at the same concentration of CMP-NANA (2 μg/ml) used in the growth medium. Regulation at the level of C3, without decreased C4 binding, may be explained by the fact that near-maximal factor H binding was observed when organisms were grown in medium containing 2 μg/ml CMP-NANA (Fig. 6).

Functional correlates of MBL binding to 24-1 and 24-1 NANA

We examined the ability of MBL to kill gonococci when organisms were incubated under the three opsonization conditions described above. Preopsonization of organisms with MBL-MASP, followed by addition of MBL-deficient serum (absorbed) resulted in 67% killing of 24-1 and 18% killing of 24-1 NANA (grown in 2 μg/ml CMP-NANA; filled bars, Fig. 7), but no killing of 24-1 NANA grown in higher concentrations of CMP-NANA. Reconstitution of MBL-deficient serum (absorbed) with MBL-MASP, before addition of bacteria (postopsonization), resulted in no killing of 24-1 or 24-1 NANA. Incubation of bacteria with 10% MBL-deficient serum (unabsorbed; Ab-dependent classical pathway activation intact) resulted in 100% killing of 24-1 and 30% killing of 24-1 NANA (grown in 2 μg/ml CMP-NANA), but no killing when 24-1 was grown in medium containing higher concentrations of CMP-NANA. These data again suggest that complement activation induced by MBL that results in bacterial killing can occur only when bacteria are preopsonized with pure MBL-MASP, but not when MBL-MASP is added to MBL-deficient serum immediately before addition of bacteria (postopsonization). Furthermore, in the case of sialylated gonococci, preopsonization with MBL-MASP alone is only partially effective (≤20% killing) when organisms are grown even in the presence of low concentrations of CMP-NANA (2 μg/ml).

Discussion

MBL binds to a wide array of carbohydrate structures on microbes (5, 10, 25–33, 35, 50, 58–63), and therefore is thought to constitute an important first-line defense against invading pathogens, because it activates C4 and C3 (4–7, 26, 58, 64). Although MBL deficiency can be a predisposing factor for recurrent infections (25, 64–73), the role of MBL in complement activation in vivo and its role in effecting complement-mediated bacterial killing remains undefined. MBL binding to Neisseria meningitidis and N. gonorrhoeae has been demonstrated (28, 29, 35, 74, 75). Sialylation of gonococcal and meningococcal lacto-N-neotetraose LOS structures decreases MBL binding (29, 35). Meningococci preopsonized with pure MBL-MASP possess the ability to activate and bind C4 (35). Binding of pure MBL to pathogens isolated from

![FIGURE 6.](http://www.jimmunol.org/)

**FIGURE 6.** Sialylation of gonococcal LOS influences MBL-induced complement activation and binding. *N. gonorrhoeae* strain 24-1 was grown in increasing concentrations of CMP-NANA and complement component binding to bacteria was quantified by whole-cell ELISA, except binding of factor H (added pure), which was determined by flow cytometry. Organisms were 1) preopsonized with MBL-MASP, followed by the addition of 10% MBL-deficient serum (absorbed against glutaraldehyde-fixed *N. gonorrhoeae* strain 24-1; filled bars); 2) added to 10% MBL-deficient serum (absorbed against 24-1) first reconstituted with MBL-MASP to a concentration of 3 μg/ml (postopsonized; open bars); or 3) added to unabsorbed MBL-deficient serum (Ab-dependent classical pathway activation intact; hatched bars), used as a positive control. Final MBL-MASP concentrations in all reaction mixtures was 3 μg/ml (a 10-fold excess of MBL-MASP relative to the amount of serum). The mean ± SD of one representative experiment (performed in duplicate) is shown.

![FIGURE 7.](http://www.jimmunol.org/)

**FIGURE 7.** Bactericidal activity of MBL against sialylated *N. gonorrhoeae* strain 24-1 incubated in increasing concentrations of CMP-NANA. MBL-MASP- and MBL-deficient serum concentrations are as described in Fig. 5. The mean ± SD of one representative experiment (performed in duplicate) is shown.
immunocompromised children has also been studied (76–79). Candida species, Aspergillus fumigatus, Staphylococcus aureus, and group A streptococci exhibit strong MBL binding. E. coli, Klebsiella species, and Haemophilus influenzae type b demonstrate heterogeneous binding patterns, and group B streptococci, Streptococcus pneumoniae, and Streptococcus epidermidis bind MBL at low levels. A study of MBL binding to different bacterial species that cause meningitis has also shown wide variations in MBL binding among pathogens (28). For example, encapsulated meningococci (representing 11 serogroups other than group A), Neisseria mucosa, H. influenzae type b, and group B streptococci all had low MBL binding capacities. E. coli K1, Streptococcus suis, S. pneumoniae and N. meningitidis serogroup A showed intermediate MBL binding capacities. Unencapsulated bacteria such as Listeria monocytogenes, nontypable H. influenzae, unencapsulated N. meningitidis, Neisseria cinera, and Neisseria subflava strains bound MBL well (28). This study concluded that the majority of encapsulated pathogens that cause bacterial meningitis had low MBL binding capacities, while unencapsulated strains bound MBL better. It is possible that capsule prevents binding of MBL to underlying surface structures.

The 20 gonococcal strains that we screened for MBL binding also showed marked variations in MBL binding among strains, probably a reflection of the phase variation of LOS species expressed by N. gonorrhoeae (48, 80–83). A terminal GlcNAc is not required for MBL binding to gonococci, as evidenced, for example, by MBL binding to an rfaK mutant of gonococcal strain MS11 that lacks terminal GlcNAc residues. This suggests that MBL may recognize LOS lacking terminal GlcNAc or structures other than LOS on the surface of gonococci (29). Indeed, in a recent report (84) porin B and opacity-associated protein of N. meningitidis have been shown to bind MBL.

While several pathogenic bacteria preopsonized with MBL are able to activate C4 in vitro (32, 35), we believe that preopsonization with MBL is less likely to occur in vivo. Regulatory molecules such as α2M are present in cervical secretions (85), and C1-INH is present on human spermatozoa (86) and in ovarian follicular fluid (87). Our study set out to examine the role of MBL in activating complement on the gonococcal surface and the effect of serum protein regulators, which more adequately represent conditions that these bacteria may encounter in vivo.

In accordance with prior observations showing that bacteria preincubated with pure MBL can activate complement (5, 26, 27, 31, 32, 35, 50), we too have demonstrated that preincubation of un sia lylated gonococci with MBL-MASP, followed by the addition of MBL-depleted serum as a source of complement, results in efficient complement activation and bacterial killing (Figs. 2 and 3). Preopsonization enhanced C4d (a measure of total number of C4 molecules deposited on bacteria) and C3b binding to bacteria. Preopsonization decreased IgM binding to bacteria ~2-fold, suggesting that MBL and IgM probably compete for identical or closely related structures/epitopes on the bacterial surface. This is consistent with the observation that IgM present in NHS that binds to gonococci is directed against LOS (88–90). We have also shown that an intact classical pathway is necessary for augmentation of C3b and factor Bb binding to 1291a preopsonized with MBL-MASP (Fig. 4). However, when MBL-MASP was added to the bacteria together with serum (a circumstance we termed postopsonization), no killing occurred, suggesting that the complement-activating function of MBL is regulated by other serum factor(s). C1-INH and α2M have been shown to regulate MBL-induced complement activation (36, 39–41). Both C1-INH and α2M inhibited MBL in a dose-responsive fashion when added to bacteria together with pure MBL-MASP (Fig. 5). C1-INH and α2M appeared to act synergistically to block complement activation by MBL-MASP. The combination of purified C1-INH and α2M blocked killing by MBL-MASP even when the latter was present in a relative (10-fold) excess (Fig. 5A). Concomitant binding of C4bp, seen with strain 1291a (data not shown), may also have contributed to the inability of MBL-MASP to deposit complement effectively when organisms were postopsonized. This demonstrates the role of traditional complement regulators such as C4bp, in addition to C1-INH and α2M, in contributing to down-regulation of complement under the influence of MBL.

The importance of the alternative pathway regulator, factor H, in regulating complement activation by the MBL pathway was also shown, because sialylated 24-1 (grown in medium containing 2 μg/ml CMP-NANA) survived (80%) killing, even when preopsonized with MBL-MASP (Fig. 7). In these experiments, we absorbed out strain 24-1-specific Ab, because 24-1 in the unsialylated state is exquisitely susceptible to killing by NHS containing bacteria-specific Ab. The presence of an intact Ab-dependent classical pathway would not allow the potential complement-fixing role of MBL on unsialylated organisms to be quantified. Survival increased to 100% when higher CMP-NANA concentrations were used. Maximal factor H binding was observed at CMP-NANA concentrations as low as 2 μg/ml (Fig. 6). Because factor H down-regulates the alternative pathway feedback loop, these data add to the evidence (provided by the observation of a dramatic increase in factor Bb binding to 1291a; Fig. 3) that complement activation by MBL-MASP on preopsonized gonococci may involve substantial recruitment of the alternative pathway. C3-convertases deposited by the MBL pathway on erythrocytes are particularly susceptible to regulation by C4bp and factor H, an effect that has been attributed to MBL-induced enhancement of C4bp and factor H binding to their ligands, C4b and C3b, respectively (8).

Collectively, our data suggest that MBL in NHS, where regulators are also present, usually does not contribute to complement activation on the gonococcal surface. Indeed, a very recent study (the first to our knowledge) addressed the role of MBL in C3 deposition on S. aureus, in the context of other serum components. No differences in C3 deposition was observed when organisms were incubated with hypogammaglobulinemic serum (91). Classical pathway activation mediated by Abs appears to be an absolute requirement for complement activation and gonococcal killing, as suggested previously (92–95).

Recent work has identified CR1 as the cellular receptor for MBL (13). Therefore, it is possible that MBL functions as an opsonin to mediate bacterial clearance by phagocytes, and this may be its primary role in protection against infection. Our study illustrates the importance of studying the biological function of complement proteins in a total context, in addition to isolating functional effects by using pure components, realizing that the former may be more likely to represent in vivo conditions.

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