Enhancement of Complement Activation and Opsonophagocytosis by Complexes of Mannose-Binding Lectin with Mannose-Binding Lectin-Associated Serine Protease After Binding to *Staphylococcus aureus*[1]

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Human mannose-binding lectin (MBL) is a serum protein of the innate immune system that circulates as a complex with a group of so-called MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3). Complexes of MBL-MASP2 are able to activate the complement system in an Ab and C1-independent fashion after binding of the lectin to appropriate microbial sugar arrays. We have evaluated the additive effect of the lectin pathway relative to other complement activation pathways and the subsequent effect on neutrophil phagocytosis. Complement activation in the sera of MBL-deficient individuals was studied with and without the addition of exogenous MBL-MASP. Flow cytometry was used to measure the deposition of C4, factor B, C3b, and iC3b on *Staphylococcus aureus.* Deposition of the first cleavage product of the lectin pathway, C4b, was increased using the sera of three different MBL-deficient individuals when exogenous MBL-MASP was added. Factor B was deposited in association with C4, but there was no evidence of independent alternative pathway activation. Similar enhancement of C3b deposition was also observed, with evidence of elevated amounts of C3b processed to iC3b. The increase in opsonic C3 fragments mediated by MBL was associated with a significant increase in the uptake of organisms by neutrophils. We also observed significant increases in phagocytosis with MBL-MASPs that were independent of complement activation. We conclude that MBL-MASP makes a major contribution to complement-mediated host defense mechanisms. *The Journal of Immunology,* **2002, 169: 4430–4436.**

Mannose-binding lectin (MBL)[3] is a member of a family of Ca^{2+}-dependent collagenous proteins called the collectins now recognized as playing an important role in innate immunity (1–3). It is an acute phase protein of hepatic origin that is able to bind through multiple lectin domains to repeating sugar arrays on microbial surfaces and subsequently activate the complement system (4). This activation is Ab and C1q independent and requires MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3) (5–7), which are believed to be activated after binding of an MBL-MASP complex to a sugar-rich microbial surface. The activated serine protease is then able to cleave sequentially C4 and C2 in a manner analogous to that of the C1-esterase of the classical pathway. Such cleavage results in the generation of covalently bound C4b2a complexes, which are able to function as C3 convertase enzymes. The direct consequence of this is amplified C3 cleavage and the production of opsonic C3b/iC3b fragments.

In man, the single MBL gene encoding the protein is found on chromosome 10 (8, 9). Several polymorphisms in the 5′ promoter region and exon 1 of the gene have been described and act together to give a wide range of serum concentrations (10, 11). Three single-point mutations in codons 52, 54, and 57 of exon 1 (also called variants D, B, and C) encode for amino acid substitutions that interfere with the correct oligomeric assembly and/or secretion of the protein (12–14). Each of these mutations is associated with a profound defect in the expression of biological effector functions such as complement activation.

The frequency of the variant structural gene alleles and of the three other polymorphic sites called H/L, X/Y, and P/Q differ between various population groups (10–12, 15, 16). Seven different haplotypes have been described to date, and four of these (LYPB, LYQC, HYPD, and LXPA) are associated with low levels of the protein. In many populations MBL deficiency is the most common immunodeficiency described to date, and several clinical studies have established an association between MBL deficiency and disease susceptibility (Reviewed in Refs. 17 and 18).

Although there is a clear role for MBL in the early stages of any primary immune response before specific Abs are produced (19), there have been few attempts to dissect out the contribution of the MBL-MASP pathway relative to other pathways of complement activation. Here we describe an evaluation of the in vitro enhancement of such activation after the addition of exogenous MBL-MASP to the serum of three MBL-deficient adult donors. Serum with or without MBL-MASP was incubated with the pathogenic organism *Staphyloccocus aureus* and in each donor we obtained evidence of an enhancement of complement activation attributable to the lectin. To determine the physiological relevance of this enhancement, we also measured the phagocytosis of *S. aureus* after

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[3] Abbreviations used in this paper: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; HBSS(cmf), calcium- and magnesium-free HBSS; MOI, multiplicity of infection; MFI, median fluorescence intensity.

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incubation of the organisms in serum with and without the addition of exogenous MBL-MASP.

Materials and Methods

Preparation of MBL-MASP

MBL-MASP was prepared as previously described by Kilpatrick (20). Briefly, ammonium sulfate was added to 500 g of frozen ethanol-fractionated human plasma paste (fraction B+1, equivalent to Cohn fraction I+III; donated by C. Dash, Blood Products Laboratory, Elstree, U.K.) to give 42% saturation. After dialysis, the solution was applied to a mannan-agarose (Sigma-Aldrich, Poole, U.K.) column (5 ml packed volume; Pharmacia Biotech, Uppsala, Sweden), and the calcium-dependent proteins were eluted with 0.01 M EDTA. The first EDTA eluate was recalcified to 0.05 M $CaCl_2$, and was applied to a second mannan-agarose column and eluted with 0.1 M mannose. The concentration of MBL was determined by ELISA and sample homogeneity was verified by nonreducing SDS-PAGE using a 3–10% polyacrylamide gradient gel and silver staining. Bands observed on silver staining were confirmed to be higher order oligomers of MBL by immunoblotting and ECL detection according to the method of Lipscombe et al. (21). MBL prepared in this manner is known to be noncovalently associated with MASP (6).

Because ficolins have been shown to bind MASP and activate complement (22), the possibility of ficolin contamination of the MBL preparation was a concern. This was addressed by dot blot analysis of both the MBL preparation and purified p35 ficolin (kindly provided by T. Fujita, Fukushima Medical University, Fukushima, Japan). MBL (at 830 μg/ml) and p35 ficolin (at 100 μg/ml) were separately blotted on Hybond P membranes (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and unoccupied sites on the membrane were blocked by incubation for 2 h with SuperBlock (Pierce Chemical, Perbio Science, Tattenhall, U.K.). The membranes were then washed three times with TBS containing 0.001% Tween before probing with either monoclonal anti-p35 (clone GN-4 at 1 μg/ml; courtesy of T. Fujita) or monoclonal anti-MBL (clone 131-1 at 1 μg/ml; State Serum Institut, Copenhagen, Denmark). After incubation for 2 h at room temperature on a shaking table, the membranes were washed three times with TBS containing 0.001% Tween. The membranes were then incubated (2 h at room temperature) with goat anti-mouse Ig-HRP conjugate (Sigma-Aldrich) diluted 1/1000 in TBST. The membranes were again washed three times with TBS containing 0.001% Tween and developed with the ECL reagent (Amersham Pharmacia Biotech). These analyses revealed no traces of ficolin in the membrane-bound MBL-MASP preparation when purified ficolin clearly bound to the membrane under the same conditions, as detected by a strong signal with the specific anti-ficolin Ab (data not shown).

Human sera

Serum was available for study from three MBL-deficient donors (RC, NS, and DM). The MBL haplotypes and serum MBL levels of these individuals were as follows: donor RC, HYPD/LYPA (150 ng/ml); donor NS, LYPB/LXPA (150 ng/ml); and donor DM, LYPB/LYPB (<150 ng/ml). The median level in the U.K. population is 1600 ng/ml (12). Levels of MBL were determined using a commercially available ELISA kit (State Serum Institute).

Bacterial strains

S. aureus strain 6571 was obtained from the National Collection of Type Cultures (Colindale, U.K.) and was stored on beads at −70°C. Organisms from beads were cultured overnight at 37°C in 6% CO_2 on blood agar and subcultured once before use. Immediately before each experiment, organisms were suspended in HBSS containing 0.05 M $CaCl_2$ and 0.05 M $MgCl_2$ (Life Technologies, Paisley, U.K.) at 1–3 × 10^8 organisms/ml (measured as an absorbance of 0.5 at 540 nm). We have previously shown that this organism binds a high level of MBL (23).

C4 and Bb deposition on S. aureus

The binding of C4 was performed by a modification of our previous method (23, 24). Briefly, 50-μl aliquots of organisms at a concentration of 1–3 × 10^8 organisms/ml were spun in Eppendorf tubes at 9470 × *g* for 2 min. In the initial experiments, supernatants were removed and the pellets were resuspended in 25 μl of MBL-deficient serum diluted to 10% with HBSS containing 0.05 M $CaCl_2$ and 0.05 M $MgCl_2$, to which were added different amounts of purified human MBL (to give 0.3, 0.6, 1.2, 2.5, 5, and 10 μg/ml final concentration). The mixtures were then incubated for 5 min at 37°C. The reaction in each tube was stopped by adding 1 ml of ice-cold calcium- and magnesium-free HBSS (HBSS(cmf)) containing 1% paraformaldehyde, and pelletted organisms were washed twice in 200 μl of HBSS. A mixture of FITC-conjugated anti-MBL (clone 131-1; State Serum Institute; conjugated as described by Johnson and Holborow (25)) and biotinylated anti-C4d (Quidel, San Diego, CA; supplied by Technoclone, Dorking, U.K.) was added to give a final concentration of 4 μg/ml for each, and the mixture was incubated for 45 min on ice. Biotin conjugation of anti-C4d was conducted using biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma-Aldrich) at a ratio of ester to Ab of 7.5:1. Streptavidin PE-Cy 5 (BD PharMingen, Cowley, U.K.) at a concentration of 0.66 μg/ml was added, and the organisms were incubated as before. Suspensions were spun at 9470 × *g* for 2 min. The supernatants were removed and the pellets were washed with 200 μl of HBSS(cmf). The samples were then fixed using equal volumes of PBS/Facs-fix. Flow cytometry was performed on a FACSCaliber at low flow rates using CellQuest software (BD Biosciences, Cowley, U.K.). Organisms were selected on the basis of size and granularity and were analyzed by two-color flow cytometry. Binding of C4 was evaluated on at least three occasions for each donor. A negative control comprising organisms processed in the same way but in the absence of serum and MBL-MASP was included in every assay.

In subsequent experiments, after the initial centrifugation of organisms the supernatants were removed and the pellets were resuspended in 25 μl of either 10% MBL-deficient serum or 10% MBL-deficient serum supplemented with 5 μg/ml purified MBL-MASP. The mixtures were then incubated for 30 s or 1, 2, 3, 5, or 10 min at 37°C. Thereafter the reaction was stopped in each tube and the assay completed as above.

In certain experiments the binding of Bb was determined at the same time as C4b binding. Sample mixtures were incubated as described above for up to 10 min at 37°C in the presence or absence of 5 μg/ml of purified MBL-MASP. The reaction was stopped as before by ice-cold HBSS(cmf) 1% paraformaldehyde, and the preparations were washed twice in 200 μl of HBSS(cmf). A mixture of FITC-conjugated anti-Bb (Quidel; conjugated as described by Johnson and Holborow (25)) and biotinylated anti-C4d (Quidel) was added at a final concentration of 4 μg/ml for each, and incubation was allowed to proceed for 45 min on ice. Streptavidin PE-Cy 5 (BD PharMingen) at a concentration of 0.66 μg/ml was added and the organisms were incubated as before. Suspensions were spun at 9470 × *g* for 2 min, and the pellets were washed as described above. Samples were fixed and analyzed by flow cytometry as described above.

iC3b/C3b deposition on S. aureus

iC3b and C3b fragments were bound to organisms when sample mixtures were incubated as described above for up to 20 min at 37°C. The reaction was stopped as before using ice-cold HBSS(cmf) containing 1% paraformaldehyde, and the organisms were washed twice in 200 μl of HBSS(cmf). Into separate tubes corresponding to each time point were added 25 μl of mouse anti-human iC3b (tissue culture supernatant used at 1/20 dilution) or 25 μl of mouse anti-human C3b (tissue culture supernatant used at 1/50 dilution) (both reagents (26) were kindly supplied by Sunita Gulati, Maxwell Finland Laboratory for Infectious Diseases, Boston, MA). The mixtures were then incubated for 45 min on ice. A preparation of FITC-conjugated $F(ab')_2$ of goat anti-mouse immunoglobulins (DAKO, High Wycombe, U.K.) was added at a final concentration of 25 μg/ml to each sample, and the mixture was incubated for 40 min at 4°C. Samples were then fixed and analyzed by flow cytometry as described above.

Opsonophagocytosis of S. aureus

S. aureus was FITC labeled as previously described (27). FITC-labeled *S. aureus* was stored in 10% glycerol broth in 50-μl aliquots at −70°C, and before use the concentration was evaluated using the Miles and Misra technique (28). Neutrophils were purified on a Ficoll/Histopaque gradient as described previously (29) using 10 ml of heparinized whole blood from a single donor. Phagocytosis was evaluated using a multiplicity of infection (MOI) of 5 organisms:1 neutrophil.

Fifty-microliter aliquots of FITC-labeled organisms at a concentration of 1 × 10^7 organisms/ml were transferred to sterile 96-well plates (Immulon 2; Dynex Technologies, Chantilly, VA). Twenty-five-microliter volumes of HBSS containing 0.05 M $CaCl_2$ and 0.05 M $MgCl_2$, 10% MBL-deficient serum, or 10% MBL-deficient serum supplemented with 5 μg/ml MBL-MASP were added to separate wells and the mixture was incubated for 1, 3, or 5 min at 37°C on a shaking table. In every experiment, serum was always diluted with HBSS containing 0.05 M $CaCl_2$ and 0.05 M $MgCl_2$. The reaction was stopped at each time point by adding 200 μl of ice-cold HBSS(cmf), and the mixtures were spun for 10 min at 1500 × *g*. The supernatants were discarded and the pellets resuspended in 50 μl of ice-cold HBSS(cmf). Neutrophils (2 × 10^6/ml) in 50-μl aliquots were added and the mixtures were incubated for 10 min at 37°C on a shaking table. After the addition of 100 μl of ice-cold HBSS(cmf), the samples

were spun for 10 min (1500 × *g*). The pellets were resuspended in 100 μl of PBS and fixed using 100 μl of Cell-fix (BD Biosciences) diluted 1/10 in PBS before analysis by flow cytometry. Uptake of the FITC-labeled *S. aureus* was evaluated by determining the median fluorescence intensity (MFI) of the entire neutrophil population. To estimate the proportion of internalized as opposed to adherent bacteria, extracellular fluorescence was quenched by adding trypan blue (2 mg/ml) for 10 min. The samples were then reanalyzed by flow cytometry, and MFI values were determined (30). In one series of experiments, neutrophils were purified from four different healthy adult donors to minimize any donor-specific effect. In another series of experiments, heat-inactivated serum (56°C for 30 min) was used in addition to unheated serum, and the organisms were analyzed for evidence of deposition of MBL-MASP, C4b, and iC3b using methodology previously described (24) and appropriate specific Abs as outlined above.

Statistical analysis

The MFI data for C4, Bb, C3b, and iC3b were handled in the following manner to allow comparisons across multiple experiments. The maximum MFI obtained within an experiment was assigned a value of 100%, with all other MFIs within the same experiment expressed as a percentage of this maximum. To compare the binding of C4, factor B, C3b, and iC3b and phagocytosis of organisms over time, the area under the curves for each experiment were calculated using Sigmaplot 2001 software (SPPS, Chicago, IL). The mean curve areas were compared by paired *t* test using SPSS 10 software. To obtain "threshold" information on the response of C4 binding and phagocytosis in relation to MBL concentration, individual data points (with and without MBL) were compared by nonpaired *t* tests using SPSS 10 software.

Results

Early complement component activation on S. aureus

We investigated the influence of bound MBL-MASP complexes on the activation of complement on *S. aureus* by measuring the binding of complement components C4 and factor B over time. Organisms were incubated with 10% serum from three different MBL-deficient donors, to which were added purified MBL-MASP complexes (5 μg/ml).

As shown in Fig. 1, C4 deposition was detected by 0.5-min incubation in serum. The amount of C4 appeared to be enhanced by MBL-MASP at this time point and all subsequent time points up to 10 min. C4 deposition was maximal at 5 min in both the presence or absence of MBL-MASP, after an initial peak at 1 min. After 5 min, the amount of C4 declined, this process being more marked in the presence of MBL-MASP. To compare the amount of C4 deposited over the course of the experiment, the area under the curve for each separate experiment with and without MBL-MASP was calculated and compared by paired *t* tests. MBL-MASP significantly enhanced the total amount of C4 deposited over 10-min incubation in serum ($p = 0.002$; Fig. 1).

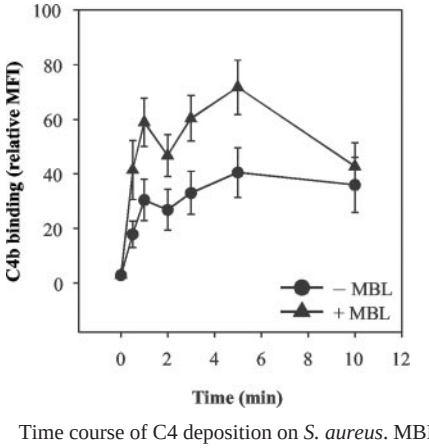

FIGURE 1. Time course of C4 deposition on *S. aureus*. MBL-MASP (5 μg/ml) was added to MBL-deficient serum from three different donors and the C4 deposition was determined by flow cytometry at different time points. Binding of C4 is expressed as relative MFI based on the maximum MFI obtained in each experiment. MBL-MASP significantly increased the deposition of C4 over the course of 10 min ($p = 0.002$, paired *t* test of curve areas, $n = 12$). ●, 10% MBL-deficient serum; ▲, 10% MBL-deficient serum supplemented with 5 μg/ml MBL-MASP. Error bars indicate ± SEM.

To determine the dosage effect of MBL-MASP on C4 activation, organisms were incubated for 5 min in MBL-deficient serum, to which were added differing concentrations of the MBL-MASP preparation (0–10 μg/ml). To highlight differences in C4 deposition at different MBL-MASP concentrations, we calculated the percentage of organisms positive for C4. We found that using MBL-MASP at a concentration of 0.6 μg/ml or above resulted in a significant enhancement of C4 deposition ($p < 0.05$, unpaired *t* test). C4 deposition appeared to be maximal at 5 μg/ml MBL-MASP (data not shown).

C4 deposition precedes the generation of the classical pathway C3 convertase C4b2a. This enzyme cleaves C3 to generate C3a and C3b fragments. In addition to their opsonic role, the latter are also an integral part of the amplification loop of the alternative pathway in which they form a complex with factor B. This leads to the generation of C3bBb complexes, which also when surface bound express C3 convertase activity. The relationship between C4 and Bb deposition was analyzed at three time points by flow cytometry using 10% serum from an MBL-deficient donor (RC) or the same serum to which was added exogenous MBL-MASP (final concentration 5 μg/ml).

In the absence of MBL-MASP, ~35% of the organisms showed evidence of C4 binding after 30 s, and the addition of exogenous MBL-MASP increased this to >60% (Fig. 2). At this time, there was negligible Bb deposition on the organism. When samples were analyzed after 1 min, the data were similar.

When samples were removed and analyzed after incubation for 3 min, ~25% of the organisms showed evidence of both C4 and Bb binding in the absence of MBL-MASP. However, when the MBL-deficient serum was supplemented with MBL-MASP, >50% of organisms stained positively for both ligands (Fig. 2). We did not observe factor B deposition on organisms that had not already bound C4.

Interestingly, although MBL-MASP increased the percentage of organisms with Bb attached, the total amount of Bb deposited on *S. aureus* by 10 min, calculated as the area under the Bb deposition curves, showed that overall, MBL-MASP did not significantly increase factor B deposition ($p > 0.05$, paired *t* test of curve areas, $n = 5$).

C3b/iC3b deposition

Because the conversion of opsonic C3b to iC3b may be relevant to the biological response to an organism, we studied this transition by flow cytometry. *S. aureus* was incubated with 10% serum from an MBL-deficient donor (RC) or with the same serum supplemented with 5 μg/ml MBL-MASP. C3b deposition was detected after 1-min incubation with serum (Fig. 3*A*), and as for C4, there was an enhancement of deposition in the presence of MBL-MASP that was maintained over the course of 20 min. C3b deposition was maximal at 3 min, followed by a reduction in the amount bound.

In contrast, iC3b deposition was delayed compared with C3b deposition, with little detectable binding up to 3 min of incubation in serum (Fig. 3*B*). After this time point, iC3b was detectable and appeared to be enhanced in the presence of MBL-MASP. To compare the total amounts of C3b and iC3b deposited over the time course, curve areas were calculated and compared by paired *t* test. MBL-MASP significantly enhanced the deposition of both C3b and iC3b ($p < 0.05$). However, the ratio of C3b to iC3b did not differ significantly in the presence or absence of MBL-MASP ($p > 0.05$).

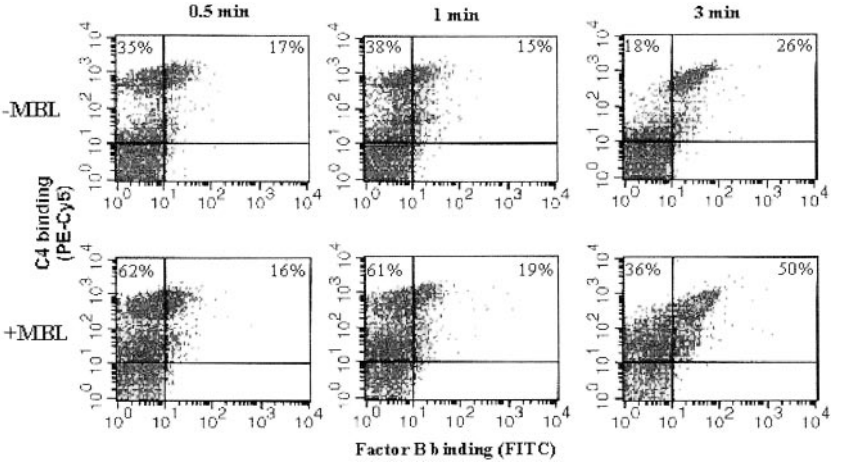

FIGURE 2. Deposition of C4 and factor B on *S. aureus* by flow cytometry. Organisms were incubated with 10% MBL-deficient serum (donor RC) or MBL-deficient serum supplemented with MBL-MASP (5 μg/ml). Reactions were stopped at 0.5, 1, and 3 min with ice-cold buffer, and the deposition of C4 and factor B was determined by dual-color flow cytometry. The plots shown are representative density plots of C4 against factor B at the different time points from 10 different experiments.

Uptake of organisms by phagocytes

To investigate the influence of MBL-MASP on opsonophagocytosis, FITC-labeled *S. aureus* preparations were incubated for 1 min with 10% MBL-deficient serum (RC) supplemented with MBL-MASP over a range of concentrations (0–5 μg/ml). After the addition of neutrophils, opsonophagocytosis was measured by flow cytometry.

As shown in Fig. 4, the addition of increasing MBL-MASP concentrations resulted in an enhancement of opsonophagocytosis compared with that observed using MBL-deficient serum alone (unpaired *t* test, $p < 0.05$ for all concentrations tested). The differences were greatest when MBL-MASP was used at concentrations between 1.25 and 2.5 μg/ml, values close to the median concentration of the protein (1.63 μg/ml) found in British Caucasians lacking MBL mutations (12).

When FITC-labeled bacteria were incubated with 10% MBL-deficient serum (RC) and 10% heat-inactivated MBL-deficient serum for different periods of time (0–3 min), it was found that the addition of MBL-MASP (5 μg/ml) resulted in an increase of opsonization in both unheated and heat-inactivated serum, with major differences apparent within 1 min (Fig. 5). The maximum level of phagocytosis coincided with the opsonization time that gave the maximum amount of C3b deposition.

The addition of exogenous MBL-MASP to the MBL-deficient serum significantly increased opsonophagocytosis as evaluated by

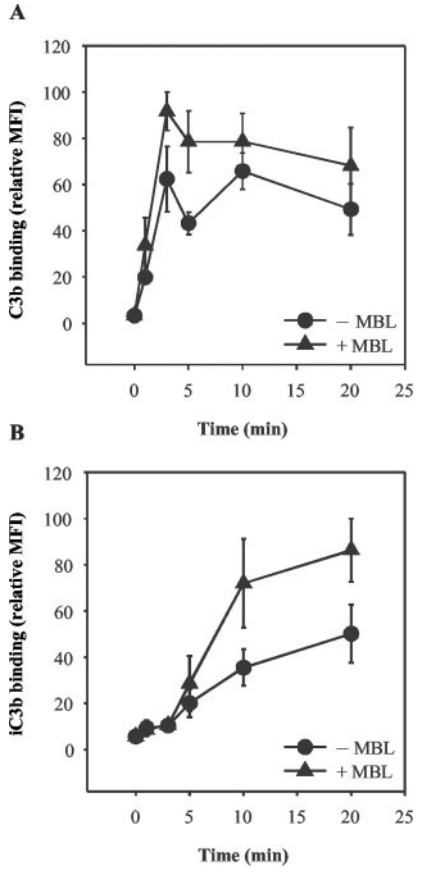

FIGURE 3. The deposition of C3b and iC3b on *S. aureus* NCTC657 using 10% serum from an MBL-deficient donor (RC). Exogenous MBL-MASP was added to give a final concentration of 5 μg/ml, and the reactions were stopped at 0, 1, 3, 5, 10, and 20 min with ice-cold buffer. The binding of C3b (*A*) and iC3b (*B*) was determined separately by flow cytometry. Binding of both moieties is expressed as relative MFI based on the maximum MFI obtained in each experiment. MBL-MASP significantly increased the deposition of C3b and iC3b over the 20-min time course ($p = 0.019$ (C3b) and 0.033 (iC3b), paired *t* test of curve areas, $n = 4$). ●, 10% MBL-deficient serum; ▲, 10% MBL-deficient serum supplemented with 5 μg/ml MBL-MASP. Error bars indicate ± SEM.

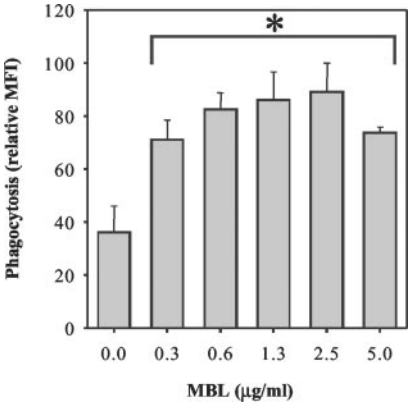

FIGURE 4. The influence of MBL-MASP concentration on opsonophagocytosis of *S. aureus*. Organisms were incubated for 5 min with 10% MBL-deficient serum (donor RC), to which exogenous MBL-MASP had been added at differing concentrations. After washing, the organisms were added to suspensions of neutrophils at an MOI of 5:1. Phagocytosis was quantified by flow cytometry and expressed as the relative MFI of the neutrophil population. Histogram bars are shown as mean MFI ± SEM ($n = 3$). The bars marked with an asterisk indicate the concentrations of MBL-MASP giving a significant enhancement of phagocytosis ($p < 0.05$, nonpaired *t* test).

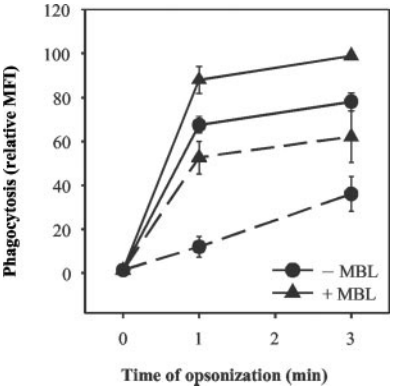

FIGURE 5. Opsonophagocytosis of *S. aureus*. FITC-labeled *S. aureus* was incubated with MBL-deficient serum supplemented or not with MBL-MASP (5 μg/ml) for different time periods. After washing, organisms were added to a suspension of neutrophils at an MOI of 5:1 and phagocytosis was stopped after 10 min. The uptake of organisms by neutrophils was analyzed by flow cytometry and expressed as relative MFI. Continuous lines show experiments using MBL-deficient serum, with the dashed lines showing experiments using the same serum heat inactivated at 56°C. ●, 10% serum from MBL-deficient donor; ▲, 10% serum from MBL-deficient donor supplemented with MBL-MASP (5 μg/ml). Data plotted as mean MFI ± SEM ($n = 3$).

comparing the curve areas by paired t test ($p = 0.015$ and 0.003 for unheated serum and heat-inactivated serum, respectively). Whereas there was the expected deposition of both C4b and iC3b on organisms incubated with unheated serum, there was no evidence of either moiety on the surface of organisms incubated with heated serum (data not shown).

The patterns of opsonophagocytosis observed after the addition of trypan blue, though variable between different donors, suggested that the MBL-MASP complex both enhanced the attachment of organisms to neutrophils and promoted internalization (data not shown).

Discussion

Over the last decade it has become clear that there is a third pathway of complement activation known variously as the MBLectin pathway, the MBL pathway, the lectin pathway, or the MBL/MASP pathway. The details of this pathway continue to emerge but essentially involve a circulating complex of the serum lectin MBL and at least three MASP enzymes together with a related truncated protein (mAp19) (31, 32). The major pathway involves MBL binding to repeating sugar arrays and activated MASP-2 cleaving its substrates, C4 and C2, to generate the C4b2a complex. Thus, the C3 convertase of the lectin pathway is generated by a mechanism that is both Ab and C1 independent.

The precise relationship of the MBL pathway to the classical and alternative pathways remains unclear, but both a MASP analog and a circulating lectin (but lacking an associated collagenous region) have been described in the Japanese ascidian *Halocynthia roretzi* (33). There is also evidence for C3-like sequences in a sea urchin cDNA library (34), suggesting that some invertebrates have a primitive complement system. A primitive MBL/MASP-activated complement system may have arisen in organisms ancestral to both chordates and modern tunicates (35). This system has been retained and refined over 400 million years of vertebrate evolution and, in mammals, MBL appears to be ubiquitous.

Two general hypotheses have been proposed for the role of human MBL. One of these envisages a major role for the protein during early life after the decay of maternal Ab but before the development of a protective Ab repertoire in the host. The presence of an effective MBL/MASP system would help to fill this "window of vulnerability" as proposed by Super et al. (36). An alternative hypothesis is that MBL/MASP is important on primary contact with an organism by providing humoral defense in the days before Ab appears—so-called "ante-Ab" (19).

There are clearly common aspects to all three complement activation pathways, and a dissection of their relative contributions in any one individual would be of interest. For the present investigations, we elected to use *S. aureus*, a microorganism frequently isolated in the context of disease but also commonly found in healthy individuals. Several studies have shown that the humoral response to this organism is complex (37–42). A wide spectrum of Abs are generated against multiple epitopes, and a proportion of these, probably differing from individual to individual, could be expected to activate the classical pathway of complement. In this report we have attempted to evaluate the "added value" of the MBL-MASP system by studying complement function in the serum of three MBL-deficient (but generally healthy) individuals, to which exogenous MBL-MASP was added. Thus, each individual, whatever their background levels of complement fixing Abs to the organism, acted as their own control.

The first cleavage product of MBL-MASP activation is C4b and the covalent binding of this fragment to nearby surfaces can be readily quantified. In the present study, MBL significantly enhanced the binding of C4 to *S. aureus* over the course of 10 min in sera from MBL-deficient donors. The observation of an increase in three different donors suggests that this phenomenon is independent of the individual Ab titers against *S. aureus* Ags. The increase in C4 deposition was detectable when the lectin was present at concentrations above 0.6 μg/ml. Recently, it has been suggested that susceptibility to infection in immunosuppressed patients would occur in those with serum levels below 0.5 μg/ml (43), and we were unable to detect significant increases in C4 deposition below this concentration.

Activation of the lectin pathway will generate a C3 convertase that will produce C3b fragments, which can combine with factor B to generate the C3 convertase of the alternative pathway. We used dual-color flow cytometry to investigate the relationship between classical or lectin pathway activation (C4 binding) and the binding of factor B. The study illustrated in Fig. 2 again used the MBL-deficient donor serum to control for background deposition of C4 and factor B via the classical and alternative pathways. The addition of MBL-MASP markedly increased the proportion of organisms positive for C4 after only 1 min (79%, compared with 53% in the absence of MBL-MASP). At this time point, there was a similar proportion of organisms staining positive for factor B in the presence or absence of MBL-MASP. At 3 min, the total proportion of organisms positive for C4 in the absence of MBL had declined, but there was an increased association with factor B. In contrast, in the presence of MBL-MASP, C4 association had not decreased at 3 min and factor B association was approximately doubled. This effect of MBL-MASP on the kinetics of complement activation and the apparent loss of C4 are similar to our previous results with *Neisseria meningitidis*, in which a more rapid deposition of C4 and C5b-9 was associated with increased killing of these Gram-negative organisms in serum (24).

Despite the observation of an increased proportion of organisms that had factor B attached to them in the presence of MBL-MASP, we were unable to demonstrate an overall increase in the total amount of factor B deposited. We did not observe any association of factor B with organisms in the absence of C4, indicating that alternative pathway activation was not occurring independently of

the classical or lectin pathways, which has been observed elsewhere for *S. aureus* at this serum concentration within the time scale studied (44). Our results suggest that factor B was probably bound through the generation of C3b by the classical/lectin C3 convertase, but this did not occur at the levels or speeds required to start the amplification loop within 10 min. A limitation of the Ab system that we used to detect factor B is that we could not discriminate between factor B and Bb resulting from factor D cleavage. At the serum concentration used, the concentration of D may be limiting, such that C3bB complexes formed might not be stable, explaining why there was no difference in factor B deposition even with more C3b in the presence of MBL-MASP. In addition, properdin concentrations may also be limiting in the system that we used. Properdin would normally inhibit the cleavage of C3b to iC3b and stabilize C3bBb complexes, but there is evidence that C3b is being efficiently cleaved to iC3b on these organisms. This may also contribute to the lack of increased B deposition in the presence of MBL-MASP. Our observation that factor B was detected on organisms that had bound C4 suggests that MBL-initiated complement activation will ultimately recruit the alternative pathway amplification loop given more time at this concentration or at higher serum concentrations. Indeed, the lectin pathway may contribute substantially to the formation of the alternative pathway C3 convertase as has been proposed elsewhere (45). Further studies will be required to resolve this issue.

C3b covalently bound to microbial surfaces is converted to iC3b as a result of enzymic cleavage by factor I at two sites in the α-chain. Although there are distinct receptors for C3b and iC3b (CR1 (CD35) binds C3b, whereas CR3 (CD18/CD11b) and CR4 (CD18/CD11c) bind iC3b), the wide distribution of these receptors ensures effective opsonophagocytosis through both the C3b and iC3b routes.

In this study, we have shown that MBL-MASP enhanced the generation of C3b and iC3b on the surface of staphylococci. The appearance of C3b fragments was detectable after 1-min incubation in serum, as has been described elsewhere (44, 46), with the delay of detectable iC3b to 3–5 min. It has been shown that there are differences between bacteria in the rate of conversion of C3b to iC3b. Gordon et al. (46) found that when bacteria were opsonised in 50% pooled human serum, C3b deposition and cleavage to iC3b occurred rapidly, and that the proportions of C3b, iC3b, and C3d on *S. aureus* were 17, 64, and 19%, respectively, whereas in the case of *Escherichia coli* the proportions were 53, 44, and 2%. However, in 10% serum, a smaller fraction of the C3b bound was converted to iC3b and our results would be consistent with this observation. MBL-MASP apparently did not alter the proportion of C3b converted to iC3b overall; it simply increased the amount of C3b deposited, which led to an increased amount of iC3b. There was some suggestion from our results that this might not be the case at all time points, and particularly at 10 min there appeared to be substantially more iC3b in the presence of MBL-MASP, when the quantities of detectable C3b were quite similar. The consequences of altering the balance between C3b/iC3b are difficult to predict. However, one possibility is that such changes may alter the pathways through which microorganisms can interact with phagocytes. This may influence subsequent inflammatory pathway activation and thereby influence the onset and progression of infections (24, 47–49).

We determined the effect of increased complement activation on the phagocytosis of *S. aureus* by neutrophils. We observed that the binding and internalization of organisms incubated with MBL-deficient serum for short time periods could be enhanced by the addition of purified MBL-MASP in a dose-dependent manner. This correlated with the increased deposition of C3 that we observed in the presence of MBL-MASP complexes. Interestingly, we also observed an increase in phagocytosis with MBL-MASP when the serum was heat inactivated and there was no evidence of concomitant deposition of either C4b or C3b. This suggests that MBL-MASP may have an intrinsic effect on phagocytosis that is not linked to its ability to enhance complement activation. The mechanisms by which MBL-MASP complexes may influence phagocytosis are controversial. It has been reported to act as a direct opsonin (50, 51) and as an agent that modifies the efficiency of other pathways, such as Ig-mediated or CR1-mediated phagocytosis (52, 53). Because our experiments were not performed in the absence of serum, it is not possible to determine whether the influence of MBL-MASP complexes was independent of other factors. However, the use of heat-inactivated serum in which complement activation did not occur suggests the possible involvement of Ig, as has been suggested by ourselves and others (49, 52–54).

One caveat applicable to all of the findings reported here is that MBL is not the sole activator of the MASP family. Matsushita and colleagues have recently shown that both L-ficolin/p35 (22) and H-ficolin (Hakata Ag) (55), serum lectins with collagen-like and fibrinogen-like domains, are able to activate complement through an association with MASP-1, MASP-2, and MAp19, which functionally parallels the MBL-MASP system. It seems likely that there is a competition between MBL and ficolin for the various serum proteases of the MASP family, and our results would be invalid if the exogenous MBL added in the experiments described was contaminated with ficolin. However, our dot blot analysis found no evidence to suggest this. Furthermore, the purification procedure used to isolate MBL involved the use of 1) EDTA and 2) mannose to displace the lectin from affinity columns, and these characteristics also argue against ficolin contamination. The binding of ficolins is apparently not Ca^{2+}-dependent, and neither H-ficolin nor L-ficolin bind to mannose. However, the serum of the MBL-deficient donor would be expected to contain both ficolins (median concentration of L-ficolin, 3.7 μg/ml; concentration range for H-ficolin, 7–23 μg/ml (56)), and therefore the background activation observed in all MBL-deficient donors studied would represent a summation of Ab-mediated classical pathway activation and ficolin-mediated activation. Further clarification of the functional interactions between the two lectins and the various MASPs is urgently required if we are to fully understand the complexities of the human complement system.

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