Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway

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Abstract

The complement system is an important part of the innate immune system. Three pathways, the classical, the alternative and the lectin pathway, lead to the cleavage of complement factor C3, a central event in the activation of the complement system. We investigated the deposition of C3b (solid-phase C3 activation product) on a mannan-coated surface at high concentration of human serum (17%). At these conditions, mannan-binding lectin (MBL) promoted the activation of C3 through the combined action of MBL-associated serine protease (MASP)-1 and MASP-2 without appreciable involvement of the alternative pathway. In serum depleted of MASP-1, MASP-2 and MASP-3, we observed synergetic effect of reconstitution with MASP-1 and MASP-2. This was inhibited by MASP-3. No C3b deposition was observed with C2- or C4-depleted serum. Depletion of factor B had no effect on the MBL–MASP-promoted C3b deposition. Our results demonstrate a function of the orphan protease MASP-1 by providing evidence that this enzyme collaborates with MASP-2 in the generation of C3 convertase, a process observable at high serum concentration, but not at low serum concentration.

Introduction

Nineteen years ago, Ikeda et al. (1) reported that rat mannan-binding lectin (MBL) (then termed mannan-binding protein) was capable of activating the complement system, presumably via the classical pathway enzymes, C1r and C1s (2). But MBL was also reported to enhance complement activation by Salmonella montevideo through the alternative pathway (3). The present view is that the third pathway, termed MBL or lectin pathway, is initiated by the binding to targets of complexes between MBL-associated serine protease (MASP)-2 and either MBL or one of the ficolins (4–7). Although MASP-1 has been shown to activate C2 and C3 (8, 9), the latter at low efficiency, there seems currently to be little enthusiasm for a place for this protein in the complement system, and the function of MASP-3 (6, 10) remains enigmatic. The complexes, MBL–MASP or ficolin–MASP, recognize patterns of carbohydrates (11) or acetylated compounds (12–14), respectively, presented by the target. The central importance of MASP-2 was deduced from experiments showing the generation of C3 convertase in a system consisting of recombinant MBL (rMBL), recombinant MASP-2 (rMASP-2) and purified C4 and C2 (15, 16). MASP-1 purified from human plasma cleaves C2, show no activity towards C4 and has repeatedly been reported to cleave C3, albeit at low efficiency (8, 9, 17). It is characteristic for the studies of the lectin pathway that serum generally has been used at high dilution (1% or less) or purified components or recombinant proteins have been employed.

We wished to investigate the activation at high serum concentration in order to approach physiological conditions and also in consideration of the reports on involvement of the alternative pathway, the activity of which is greatly reduced at serum concentrations of >10%. For activation we used a mannan surface in order to eliminate interference from the ficolins.

Methods

Depletion of complement components

Serum was recovered, after coagulation for 1 h at room temperature, from blood drawn from donors of various MBL genotypes after informed consent.

¹He died of cancer on June 1st 2006. He will be sorely missed both as a beloved friend and most capable colleague.

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MBL-deficient human serum from B/B genotype donors was depleted at 4°C for residual MBL and anti-mannan antibody by passage of 5 ml serum through a 1.5-ml column of mannan Sepharose beads prepared by coupling mannan, purified from *Saccharomyces cerevisiae* (18), to CNBr-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) at 10 mg mannan ml⁻¹ beads, as described by the manufacturers. The MBL level was by this procedure lowered from 19 ng ml⁻¹ to undetectable and the anti-mannan antibodies were depleted by <90% as determined by development of wells with anti-human Ig antibodies. The MBL-depleted serum was aliquoted, frozen at −80°C, and used for all the following depletions of other complement components. The various antibodies (see below) were coupled to CNBr-activated Sepharose 4B (except for anti-MASP-2 where divinyl sulphone-activated Sepharose was used, since this antibody was less efficient when coupled to CNBr-activated beads) in citrate buffer, pH 7.5, at 2 mg IgG ml⁻¹ for 16 h at 4°C. All columns were washed with 0.1 M glycine, pH 2.5, and equilibrated with barbital buffer [4 mM Barbital, 145 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.05% (v/v) Tween 20, 0.05% (w/v) Na₃Cit, pH 7.4]. Unless otherwise stated, serum was diluted 2-fold in the barbital buffer prior to loading. After wash with barbital buffer, bound proteins were eluted with 0.1 M glycine, pH 2.5, into 1 M Tris buffer (5% of the total volume), pH 8.5, and dialyzed against barbital buffer. All depletions were performed at 4°C.

MASP-2 was depleted by passage through a rat anti-human MASP-2 [MAb 8B5 (19)] column. MASP-2 + MAp19 were depleted by passage through a column with antibody reacting both with MASP-2 and MAp19 [MAb 6G12 (19)]. Depletion for MASP-2 was analyzed by a sandwich MASP-2 time-resolved immunofluorometric assay (TRIFMA) (19) and by SDS-PAGE of samples corresponding to 1 μl serum followed by western blotting and development with biotinylated MAb anti-MASP-2, 1.3B7 (20) (1 μg ml⁻¹). HRP-labeled streptavidin (DakoCytomation, Glostrup, Denmark), diluted 1000-fold, and enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL, USA). Signals were recorded with a cooled CCD camera (Image Station 1000, Kodak, New York, NY, USA).

MASP-1 + MASP-3 were depleted by passage through a mouse anti-human MASP-1/3 column prepared by coupling a mixture of antibodies against the shared A-chain of MASP-1 and MASP-3 [MAbs 1E2, 2B11 and 2D3 (21)]. No assay specific for MASP-1 is available, but assuming no preference for MASP-1 or MASP-3, the extent of depletion was evaluated by MASP-3 assay (22) and by the development of SDS-PAGE western blots with polyclonal rabbit anti-human MASP-3 and HPR-labeled goat anti-rabbit IgG antibody (6).

C2 was depleted by passage through a mouse anti-human C2 antibody column (Hyb 050-05-02, AntibodyShop, Copenhagen, Denmark). Serum was analyzed for depletion of C2 by development of western blots with 1 μg Hyb 050-05-02 ml⁻¹, followed by detection with HRP-labeled rabbit anti-mouse antibody (DakoCytomation) diluted 2000-fold and enhanced chemiluminescence.

C4 was depleted by passage through a column of Sepharose derivatized with rabbit anti-human C4 antibody (DakoCytomation). The serum was analyzed for depletion in a complement activation assay for the MBL pathway (23), modified by doing the analysis in one step with the serum diluted in a physiological buffer without addition of C4. Briefly, mannan-coated wells were incubated with serum [reconstituted with 1 μg rMBL ml⁻¹ serum (see below)] diluted in 10 mM Tris, 145 mM NaCl, pH 7.4 [Tris-buffered saline (TBS)] containing 0.05% Tween 20 and 5 mM CaCl₂ (TBS/Tw/Ca), and incubated for 90 min at 37°C. Bound C4 was detected by biotinylated anti-C4 antibody (23) followed by 10 ng Eu⁺³-labeled streptavidin (PerkinElmer, Boston, MA, USA) in 100 μl of TBS/Tw and 25 μM EDTA. After incubation for 1 h at room temperature (RT), the wells were washed and bound europium was detected by the addition of 200-μl enhancement solution (PerkinElmer) and reading time-resolved fluorescence on a DELFIA® fluorometer (PerkinElmer).

Factor B was depleted by passage through a column of mouse anti-human factor B antibody (Hav 005-03-1, AntibodyShop) and depletion was verified by western blotting using development with 1-μg Hav 005-03-1 ml⁻¹ followed by HRP-labeled rabbit anti-mouse antibody.

C1q was depleted by passage through a column of human IgM (Sigma–Aldrich) coupled to CNBr-activated Sepharose. Depletion of C1q was analyzed in a sandwich assay based on two anti-C1q antibodies (20).

As a control for the effect of depletion of serum components, serum was passed through a Sepharose column coupled with normal rat IgG, purified from rat serum by protein G-Sepharose (GE Healthcare) affinity chromatography.

**Proteins**

rMBL, prepared according to Vorup-Jensen et al. (15), was a gift from NatImmune A/S, Copenhagen, Denmark. The post-translational modifications and the biological activity of this preparation are indistinguishable from those of plasma-derived MBL (25). For reconstitution, 1 μg rMBL ml⁻¹ serum was used.

MASP-1 was purified by diluting serum with an equal volume of dissociation buffer (10 mM Tris, 0.05% Tween 20, 2 M NaCl, 20 mM EDTA, pH 7.4), and passage through the anti-MASP-1/3 column at 4°C. Under these conditions, the MBL–MASP and ficolin–MASP complexes are dissociated. The column was washed in dissociation buffer followed by TBS, and bound proteins (in the following referred to as MASP-1/3 eluate) were eluted with 0.1 M glycine, pH 2.5, containing 0.01% Tween 20. The fractions were neutralized, as described above, and the fractions containing MASP-1 and MASP-3 were dialyzed against TBS with 0.05% Tween 20 and 5 mM CaCl₂ (TBS/Tw/Ca). MASP-3 was removed from the MASP-1/3 eluate by incubation of 200 μl MASP-1/3 eluate with 100 μl streptavidin-labeled Sepharose beads (GE Healthcare), which had been pre-incubated with 100 μg biotinylated anti-MASP-3 antibody [38:12-3 (22)] and washed. Supernatants and eluates were analyzed in the MASP-3 assay and by western blotting. MASP-1 was visualized by rabbit anti-human MASP-1 [rabbit 64 (20)] and MASP-3 by MAb 38:12-3 as described above. Unless otherwise mentioned, 5 μl of the MASP-1 preparation was used for reconstitution.
rMASP-2 was produced by human endothelial kidney cells (HEK293) transfected with an MASP-2 construct and grown in protein-free medium, as described previously (15) except for using a construct without coding sequence for a His Taq. The supernatant, containing 7 μg MASP-2 ml⁻¹, was used without purification after dialysis against TBS/Tw/Ca. While this is less than ideal, experiments with control supernatants showed no effect on the test system. So far attempts of purifying the non-His-tagged rMASP-2 have yielded dismal results. For reconstitution, 0.5 μg MASP-2 was added per milliliter of serum, which is close to the mean concentration of MASP-2 in serum (19).

rMASP-3, produced by transfected HEK293 cells, was purified on MBL-derivatized Sepharose as described by Dahl et al. (6), and stored at 250 μg ml⁻¹ of TBS/Tw/Ca containing 0.5 M NaCl at −80°C. For reconstitution, 2 μg rMASP-3 ml⁻¹ of serum was used, which are within the normal range of the human MASP-3 concentration (22).

C2 and factor B were purified using the anti-C2 or anti-factor B columns, respectively. The concentration was determined by optical density at 280 nm, and for reconstitution, 20 μg C2 and 180 μg factor B was used per milliliter of serum. C4 (26) was purified from serum as previously described, and for reconstitution used at 600 μg ml⁻¹ of serum. All re-constitutions reflect physiological levels.

Mouse sera

Serum from C3 knockout (KO) mice (27) (kindly provided by M. Carroll, Boston) was used as a control in some assays. Serum from MBL double-KO mice (MBL-A⁻/⁻, MBL-C⁻/⁻) (28) were used to evaluate the MBL pathway. All sera were stored at −80°C. In some experiments sera were depleted for mouse MBL-A and MBL-C by incubating serum with anti-mMBL-A (MAb 4G2)-Sepharose and anti-mMBL-C for mouse MBL-A and MBL-C by incubating serum with coated with 1

Protein assay

To estimate the dilution factor arising during the depletion of sera, the total amount of protein was measured by the Bradford method using a commercial assay (Bio-Rad protein assay, Bio-Rad, Hercules, CA, USA). A standard curve was made with dilutions of non-depleted mouse serum. All re-constitutions reflect physiological levels.

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Assay for C3b deposition onto mannan

Microtitre wells (FluoroNunc, Nunc, Kamstrup, Denmark) were coated with 1 μg mannan in 100 μl of 15 mM Na₂CO₃, 35 mM NaHCO₃, 15 mM NaN₃, pH 9.6 (coating buffer) overnight at 4°C. Residual protein-binding sites were blocked by incubation with 200 μg HSA in 200 μl of TBS for 1 h at RT, followed by wash with TBS/Tw/Ca. Sera to be tested were diluted 6-fold in barbital buffer (4°C). rMBL was added (60-fold dilution of a stock of 10 μg rMBL ml⁻¹) corresponding to 1 μg ml⁻¹ of serum, and the mixtures were applied to mannan-coated wells kept on ice. C3 cleavage was allowed by incubating the plate for 20 min at 37°C with the microtitre plate in direct contact with 37°C water. After washing, the wells were incubated for 90 min at RT with 100 μl buffer (TBS/C

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MASP-1 and MASP-2 in C3 activation

The basic reagent used was MBL-deficient serum further depleted of MBL and anti-mannan antibodies. Other components were depleted by additional adsorptions. C3 activation was studied at a 17% serum concentration by measuring the deposition of C3 fragments (here referred to as C3b) onto mannan-coated microtitre well after 20 min incubation at 37°C. The high serum concentration was used to facilitate the alternative pathway (Fig. 1A). The incubation at 37°C for only 20 min was based on pilot studies on influence of temperature and time of incubation (data not shown) and chosen to avoid limitations due to exhaustion of assay components.
C3b deposition is depicted in units with reference to C3 standard curves as shown in Fig. 1(B). Without MBL added, a low but significant C3b deposition was detected in this assay. The cause of this background C3b deposition was not investigated; possibly, it was due to a direct effect of the alternative pathway. The background value was used for calculating the response after addition of MBL to the various depleted and reconstituted sera. Thus, the counts with the depleted serum without added MBL were set to 1 relative unit. All figures in Figs 1–3 uses comparable relative units.

The results shown in Fig. 1(C) were obtained with serum passed through a Sepharose column coupled with rat Ig, which was used as a control for the effect of depletions of components with Sepharose-coupled anti-complement antibodies. MASP-2 removal resulted in marked reduction in C3b deposition (Fig. 1D). All MASP-2 had been removed as analyzed by TRIFMA and western blotting, whereas the amount of MASP-3 was unchanged (results not shown). The C3b deposition was re-established by reconstitution with rMASP-2 to a physiological level. Depletion of both MASP-1 and MASP-2 lead to similar results (data not shown). Serum was depleted for MASP-1 and MASP-3 by the use of antibodies recognizing the identical A-chain of the proteins. This depletion resulted in a reduction of C3b deposition similar to that seen in MASP-2-depleted serum. No MASP-3 was detectable in the MASP-1/3-depleted serum, and 95% of the MASP-2 was still measured. No assay specific for MASP-1 is available, but MASP-1 is assumed to be depleted with the same efficiency as MASP-3 as they share the A-chain. Purification of MASP-1/3 followed by depletion of MASP-3 (Fig. 3A) made it possible to reconstitute the MASP-1/3-depleted serum with MASP-1 or MASP-3 separately or in combination (Fig. 1E). This revealed that MASP-1, but not MASP-3, is needed for efficient C3b deposition. Depletion of either C2 or C4 leads to abolished C3 cleavage (Fig. 1F and G). However, the depletion by immunoabsorption did not by
itself destroy the ability to cleave C3 as reconstitution of the serum with C2 or C4, respectively, lead to C3 cleavage. Depletion of factor B reduced the C3b deposition by \( \sim 30\% \), and this reduction was restored by reconstitution with factor B (Fig. 1H). Depletion of C1q did not influence C3 deposition as compared with the control (data not shown). MBL-sufficient serum depleted for MBL gave similar results (data not shown) as the ones presented here using MBL-deficient serum as starting material. Measurements of C3adesArg in supernatants from the mannan surfaces using a commercial ELISA kit confirmed the MASP-dependent C3 activation (results not shown).

**C3 cleavage through the MBL pathway in mouse sera**

Transgenic mice deficient in complement components made it possible to analyze the influence of MBL and MASPs in the C3 deposition assay. Mouse serum was depleted for MBL-A and MBL-C and subsequently reconstituted with 1 µg rMBL ml\(^{-1}\) serum in order to ensure the same MBL concentration, and an MBL concentration comparable to that used in the analysis of human sera. Serum from C3 KO mice was used as control (Fig. 2A). Serum from MBL KO mice led to C3b deposition when rMBL was added (Fig. 2B), confirming the assay to be MBL dependent.

**MASP-1 and MASP-2 are both needed for efficient generation of the C3 convertase**

MASP-1 purified from human serum (Fig. 3A), rMASP-2 and rMASP-3 were used for reconstituting MBL- and MASP-depleted serum, separately or in combination, and the sera were analyzed for C3b deposition through the MBL pathway (Fig. 3B). Serum was reconstituted with 1 µg rMBL ml\(^{-1}\) as in the previous experiments. Sera containing MBL-MASP-1 or MBL-MASP-2 complexes gave rise to a small increase of C3b deposition, compared with the serum only containing MBL. However, the admixture of both MASP-1 and MASP-2 together with MBL gave a significantly increased deposition of C3b with a clear synergistic effect. MASP-3 did not promote C3b deposition, alone or in combination with one or the other MASPs. On the contrary, lower C3b deposition was seen when MASP-3 was present, indicating a competitive inhibition by MASP-3. Addition of increasing amounts of MASP-1 gave rise to a dose-dependent C3b deposition (Fig. 3C), which was most profound in combination with MASP-2. Approximately 50% reduction was observed when adding MASP-3.

**C4 cleavage by MBL–MASP**

To analyze whether the decrease in C3b deposition in MASP-1/3-depleted human serum was an effect of impaired formation of the C3 convertase, C4bC2b, due to lack of C4 activation, we examined C4b deposition on the mannan surface. A serum standard was used to estimate C4b deposition units.
(Fig. 4A). Serum passed through a column coupled with normal rat Ig was used as a control (Fig. 4B). Serum depleted for MBL–MASP-2 showed cleavage of C4 only when reconstituted with rMBL and rMASP-2 (Fig. 4C). MBL–MASP-1/3-depleted serum showed C4 cleavage similar to the control serum after adding MBL (Fig. 4D). The small difference is probably due to the loss of ~5% MASP-2 in the MASP-1/3 depletion step.

Discussion

At the experimental conditions applied in this study, serum at a high concentration on a mannan surface, both MASP-1 and MASP-2 were needed for efficient cleavage of C3 through the MBL pathway. MASP-3, on the other hand, exerted inhibitory activity.

We chose to initiate C3b deposition on mannan-coated wells for recording pathway activity, thus avoiding influence of the post-C3 components, e.g. factor C5–C9. Most experiments were carried out with an MBL-deficient serum passed through mannan-derivatized Sepharose and reconstituted with rMBL in order to ensure a constant concentration of MBL, the physiological level. The same procedure depleted serum of anti-mannan antibodies. All experiments were also carried out with an MBL-sufficient serum depleted for MBL with similar results. Ficolins, recently established player in the lectin pathway of complement, do not bind to mannan-coated surfaces (31), hence have no influence on the results, and no attempts were made to remove them. As anti-mannan antibodies were removed from the serum, there should be no participation of the classical pathway. This supposition was confirmed by depleting for C1q, which did not influence on C3b deposition. We diluted the serum in calcium- and magnesium-containing barbital buffer to facilitate the calcium-dependent lectin pathway as well as the magnesium-dependent alternative pathway (32). However, as revealed by depletion of factor B, the alternative pathway seems to play a minor role at our assay conditions. In contrast, Suankratak et al. (33) reported the alternative pathway to be involved in complement-dependent hemolysis via the lectin pathway. Recently, the amplification loop of the alternative pathway by Brouwer et al. (34) reported to be required for optimal MBL-mediated opsonization of zymosan particles for phagocytosis. This study was performed by preopsonizing zymosan in 3% serum, followed by wash and incubation with purified neutrophiles. Interestingly, the early report from Schweinle et al. (3) implicating the alternative pathway in C3b deposition onto S. montevideo also used serum at low concentration (2.5%). The bacteria were preopsonized with MBL before adding serum in a calcium-free buffer (magnesium-EGTA). It is a puzzle how Schweinle et al. (3) and Suankratak et al. (33) managed to study the MBL
pathway at calcium-free conditions, since the binding of MBL to carbohydrates is calcium dependent. The low serum concentration used by several authors appears at odds with the commonly held belief that alternative pathway activity is difficult to detect at serum concentration of <10%. While important information may certainly be obtained in dissected systems, it is of some interest to try to approach more physiologically relevant conditions. We have recently reported on significant opsonizing effect of MBL in hirudin-anticoagulated full blood (35), and in the present report we use serum at 17%. In our assay setup, the C3b deposition decreased markedly upon dilution and was very low at 5% serum. At longer incubation times, significant C3b deposition is seen also at 100-fold dilution, like we and others have observed before (36, 37).

C3b deposition was impaired by depletion of MASP-2, but also, to about the same extend, by depletion of MASP-1/3 (Fig. 1D and E), indicating that MASP-2 is not the only player in generating C3 convertase by the MBL pathway. C3b deposition could be restored by reconstitution with rMASP-2 or purified MASP-1, respectively. Depletion of both MASP-2 and MAp19 showed a similar result as seen for MASP-2 depletion alone, indicating that MAp19 does not play a significant role in this setup. Without MASP-2, the conventional C3 convertase (C4bC2b) of the MBL pathway could not be formed, but C3b deposition was still observed (Fig. 3B), which indicate another pathway, e.g. direct cleavage of C3 by the MBL–MASP-1 complex (8, 9, 38). A number of studies have addressed this. Petersen et al. observed a cleavage of C3 by plasma-derived human MASP-1, but only with a 50-fold surplus of MASP-1 and long incubation at 37°C (17); however, activation could not be verified by Wong et al. (39). Serum from MASP-1/3 KO mice could not promote deposition of C3b onto a zymosan surface, suggesting that the lectin pathway was depressed in MASP-1/3 KO mice, but the experiments had to be conducted at low temperature (0°C) for this conclusion to be reached (40).

Several authors have questioned a biological role for MASP-1 in activating C3. Rossi et al. (41) found rMASP-1, expressed in insect cells, to have only a marginal activity towards C3, and Hajela et al. (42) concluded that MASP-1 can cleave hydrolyzed C3 [C3 = C3(H2O)] but not intact C3. This interpretation was supported using rMASP-1 expressed in Escherichia coli cells (43). Contrary, direct activation of C3 by MBL–MASP complexes was observed and found associated with the lower oligomeric form of MBL (MBL-I), which is mainly associated with MASP-1 and MAp19 (6). Note that these investigations, except for those on serum from KO mice, were conducted with purified components with no attempts of simulating activation on a biological surface at high serum concentration.

MASP-3 does not seem to participate in generation of the C3 convertase. However, our results indicate a role for MASP-3 as a competitive inhibitor of both MASP-1 and MASP-2, as was previously suggested to be the case regarding MASP-2 inhibition (6).

No signal was obtained when C2 or C4 were depleted from the serum, and the signal was recovered by reconstitution with these components (Fig. 1F and G), presumably since the C3 convertase (C4bC2b) could not be formed, and indicating little, if any, contribution of the alternative pathway. A C2- and MBL-deficient serum from a genetically C2-deficient patient (44) showed similar results, as also reported by others (45). This does not support the notion of direct cleavage of C3 by MBL/MASP-1. Nevertheless, a C3b deposition signal was obtained when MASP-2 was not present, and there is universal agreement that MASP-1 does not cleave C4 (Fig. 4).

Sera from MBL-A and MBL-C double-KO mice provided the opportunity to analyze the influence of these components on C3 cleavage in a transgenic system (Fig. 2). Human rMBL was used to reconstitute the mouse sera. The amount added (1 µg rMBL ml−1 of serum, which is normal level in human serum) was ~50 times less than the combined levels of MBL-A and MBL-C (29). However, preliminary experiments showed this amount to be optimal for the assay. A further implication was that human MBL functioned well with the murine components.

We also analyzed the influence of the MASPs on C4 cleavage, and found that MASP-2 is involved, whereas lack of MASP-1 and MASP-3 did not affect the C4 deposition (Fig. 4). This fits with the auto-activating nature of MASP-2 described (16, 46). However, both MASP-1 and MASP-2 influenced C3 cleavage, which combined with the results from the C2 or C4 depletions indicate that MASP-1 might be responsible for C2 cleavage. Working with pure proteins Rossi et al. (41) found that rMASP-1 do cleave C2, however, less efficiently than by rMASP-2. Chen and Wallis (47) reported that recombinant rat MASP-1 cleaves C2 almost as efficiently as MASP-2. We tried to illuminate the role of MASP-1 in activating C2. After incubation in the C3 cleavage assay, supernatant from MASP-1/3, MASP-2 or control depleted serum were compared for the amount of C2b (enzymatically active C2 fragment) generation by analyzing the supernatant for C2b by western blotting using a number of anti-C2 antibodies (commercial as well as from colleagues), but failed to obtain reproducible convincing results one way or another.

Our results point to the requirement for both MASP-1 and MASP-2 in the generation of an efficient C3 convertase. Taking this into account, the lectin pathway may be depicted as shown in Fig. 5. The evidence of MASP-1 functioning in this manner is at present circumstantial, but we have no other explanation for the influence of MASP-1. However, this means that the MBL–MASP-1 and MBL–MASP-2 complexes need to be in close proximity for an efficient formation of the C3 convertase. We suggest that the term MASP pathway is a more logical nomenclature than 'lectin pathway', by focusing on the enzymes responsible and by including activation promoted by the binding of MBL as well as the ficolin, which we now know are not lectins in the conventional definition as selective sugar-binding protein but rather recognizing patterns of acetyl groups (12). It is remarkable that the results and conclusion presented here appear quite different from those presented in a recent publication by Selander et al. (48). The setup in that study, using a surface of O antigen-specific oligosaccharides derived from Salmonella, demonstrated activation of the alternative pathway by MBL independently of MASP-1 and MASP-2. We believe that both results are valid, and demonstrate that different assay conditions reveal different routes of activation by MBL. The
alternative pathway is highly dependent on the chemical composition of the surface. In the present study, a mannan surface was used, while in the study by Selander et al., the coat was provided by a modified *Salmonella* O antigen. Clearly, further studies are needed to resolve the workings of MBL, ficolins and MASPs.

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**Abbreviations**

- C2b: enzymatically active C2 fragment
- C3b: solid-phase C3 activation product
- C4b: solid-phase C4 activation product
- KO: knockout
- MASP: mannan-binding lectin-associated serine protease
- MBL: mannan-binding lectin
- r: recombinant
- RT: room temperature
- TBS: Tris-buffered saline
- TRIFMA: time-resolved immunofluorometric assay

**References**


