Purification and Characterization of Two Mannan-Binding Lectins from Mouse Serum

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### References

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Mannan-binding lectin (MBL) is an animal C-type lectin (i.e., showing calcium-dependent carbohydrate binding) of the collectin family in which the carbohydrate recognition domains (CRDs) are attached to collagen regions (1). The mature protein is an oligomer of subunits each composed of three identical polypeptide chains of about 30 kDa united by disulfide bridges and a collagen triple helix. After binding to carbohydrates located on the surface of microorganisms, MBL activates the complement system (2). Activation occurs via C4 and C2 and is mediated by the MBL-associated serine proteases MBL-associated serine protease (MASP)-1 and MASP-2 (3, 4). MBL may also directly opsonize microorganisms for phagocytosis (5), probably by interacting with C1q/collectin receptors found on several types of cells, including macrophages. In humans, low serum levels of MBL or the presence of variant alleles have been correlated with a common opsonic defect that predisposes to recurrent infections and may be involved in recurrent abortion (6–8).

MBL is synthesized by hepatocytes and has been isolated from the liver or serum of several vertebrate species. Only one form of human MBL has been characterized, whereas two forms are found in rabbits, rats, mice, and rhesus monkeys (9). So far MBL-A has been considered to be the serum form in rodents, whereas MBL-C has been called the liver form (10). The N-terminal segment of MBL-A comprises 21 amino acid residues which, as in human MBL, include 3 cysteine residues. MBL-C has only two cysteine residues in the equivalent segment, which has led to the assumption that MBL-A forms higher oligomers than MBL-C. This has been confirmed for the rat where MBL-C forms dimers or trimers and MBL-A forms hexamers of subunits consisting of three identical polypeptide chains. Moreover, rat MBL-C dimers or trimers, unlike rat MBL-A, are reported to be incapable of activating complement (10), which has led to the assumption that this may be a general property of MBL-Cs. In mice, the differentiation between murine MBL-A (mMBL-A) and mMBL-C is complicated by their identical mobilities on SDS-PAGE in the reduced state, corresponding to polypeptide chains of 28 kDa (11). In this study, we present the purification and characterization of both forms of MBL from mouse serum.

**Materials and Methods**

**Affinity beads**

Mannose was coupled to TSK HW/75(F) beads (Tosoh, Tokyo, Japan) activated by divinyl sulfone (12). The beads were suspended in 9.1% (v/v) divinyl sulfone in 0.25 M Na2CO3, incubated for 90 min, washed with water, incubated in 10% (w/v) mannose in 0.5 M Na2CO3 (pH 11) for 24 h at room temperature, washed, and incubated for 2 h in 0.1 M ethanolamine (pH 9.0), washed, and kept in TBS.

Affinity-purified rabbit anti-mouse-IgG Ab (50 mg; see “Preparation of Abs”) was coupled to 5 ml of TSK HW/75(F) beads activated with 3% divinyl sulfone (v/v). The coupling buffer was 15 mM NaHCO3 containing 135 mM NaCl and 5% (w/v) polyethylene glycol (PEG) 20,000 (pH 8.6).

**Purification of mMBL-A and mMBL-C by carbohydrate affinity chromatography**

Pooled mouse serum (45 ml), obtained from inbred BALB/c mice or outbred NMRI mice, was mixed with an equal volume of precipitation buffer consisting of 10 mM barbital-HCl, 300 mM NaCl, 10 mM CaCl2, 15 mM
Purification of mMBL-A and -C by affinity and ion-exchange chromatography

Mouse serum (135 ml) was mixed with an equal volume of buffer consisting of 20 mM barbital-HCl, 40 mM CaCl₂, 1.0 M NaCl, 0.08% (v/v) emulphogene, 100 μM benzamidine, 100 μM iodoacetamide, and 100 μM cyclokapron (pH 7.4). The diluted serum was applied to a 70-ml TSK precolumn connected to a 70-ml mannose-TSK column pre-equilibrated with 10 mM barbital-HCl, 20 mM CaCl₂, 0.5 M NaCl, 0.04% (v/v) emulphogene, 50 μM benzamidine, 50 μM iodoacetamide, and 50 μM cyclokapron (pH 7.4; BBS-2/NaCl/Ca²⁺). After loading, the columns were washed with 10 ml of buffer followed by the same buffer with the NaCl concentration reduced to 0.15 M NaCl (BBS-2/Ca²⁺). The column was then washed for 20 min at 2000 g and the pellet was washed with BBS-1/Ca²⁺ containing 8.0% (w/v) PEG 6000. The mixture was centrifuged for 20 min at 10,000 g and the pellet was washed with BBS-1/Ca²⁺ containing 12 mM glucose ("glucose eluate"). It was then washed with 50 ml of BBS-1/Ca²⁺ and eluted with 50 ml of BBS-1/Ca²⁺ containing 25 mM mannose ("mannose eluate"). The absorbance of fractions at 280 nm was measured and relevant fractions were analyzed by rocket immunoelectrophoresis, acid/urae-PAGE, and N-terminal amino acid sequencing.

Complement activation assay

Microtier wells (FluoroNunc from Nalge-Nunc, Roskilde, Denmark) were coated overnight with 70 ng of mannann in 100 μl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 30 mM NaNO₃). Wells were blocked and reacted with 2 μg of rabbit polyclonal anti-mMBL-C antibody (Sigma, St. Louis, MO). Wells were blocked and incubated for 3 h at 37°C with human serum devoid of MBL and C1q diluted 1:90 in TBS/Ca²⁺. The solution was obtained from an MBL-deficient subject (serum MBL <10 ng/ml) and C1q was removed by chromatography on Bio Rex 70 (Bio-Rad, Bedford, MA) (17). Any remaining MBL was removed from the serum by incubating it with mannan-coupled Sepharose (15) in the presence of CaCl₂. Wells were then washed in TBS/Ca²⁺ (without CaCl₂) and incubated for 3 h with 100 μl of TBS/CA²⁺ containing 10 μg of MBL and 100 μg of rabbit anti-human C4 Ab (Dako, Glostrup, Denmark). Wells were washed in TBS/Ca²⁺ and incubated for 2 h with 10 ng europium-labeled streptavidin (Wallac, Turku, Finland) in 100 μl of TBS/Ca²⁺ containing 25 μM EDTA and 0.01% (v/v) HSA. After washing with TBS/Ca²⁺, bound europium was released into the fluid phase by incubation for 10 min with enhancement buffer (Wallac). The amount of europium in each well was assessed by time-resolved fluorometry on a Delphia fluorometer (Wallac).

Carbohydrate selectivity

Purified MBL was biotinylated with 40 μg N-hydroxysuccinimido-derivative of MBL. Microtiter wells (FluoroNunc) were coated overnight at room temperature with 13 ng of mannann in 100 μl coating buffer. Plates were blocked and washed as described above. Dilutions of monosaccharides in 50 μl TBS/Ca²⁺ were added in duplicate to the wells by means of a robotic pipetting system (Packard, Meriden, CT). Negative and positive controls consisting of TBS/Ca²⁺ or TBS/Ca²⁺-exchanged without monosaccharides were included, biotinylated MBLs at 0.15 μg/ml (mMBL-A), 0.06 μg/ml (mMBL-C), or 0.04 μg/ml (human MBL) in TBS/Ca²⁺ were added in duplicate to 50 μl of each monosaccharide. The solutions were mixed on a shaking platform and incubated overnight at 4°C. The monosaccharides tested comprised α-mannose (Man), α-methyl-α-mannose (αMeMan), L-mannosamine (ManN), N-acetyl-α-l-fucosamine (ManNac), α-glucose (Glc), α-methyl-α-glucose (αMeGlc), α-glucosamine (GlcN), N-acetyl-α-glucosamine (GlcNAc), α-galactose (Gal), α-methyl-α-galactose (αMrgal), α-galactosamine (GalN), N-acetyl- α-galactosamine (GalNAc), L-fucose (Fuc), and α-fucose (fuc); all were purchased from Sigma. All were tested at concentrations ranging from 0.184 to 100 mM. The wells were washed and developed with europium-labeled streptavidin as described above. The background was defined as the average signal of three wells incubated with biotinylated MBL in the presence of EDTA; the maximum signal was that obtained in buffer without monosaccharide. Fluorescence intensities from 10⁻⁴ to 10⁰ counts/well were obtained with 3% variance. Each monosaccharide was tested in at least five different experiments, and various combinations of five different monosaccharides were tested on the same microtiter plate to determine their individual ranking.
Estimation of mMBL by rocket immunoelectrophoresis

Rocket immunoelectrophoresis was performed as described (15) in agarose gels containing 6.7% (v/v) rabbit anti-mMBL antiserum. Samples of purified mMBL-A and two mouse sera were used as standards and controls. The mMBL-A in serum could be quantified since this precipitate was stronger and of a shape distinct from that of mMBL-C. The presence of two independent precipitates shows that separate Abs recognize mMBL-A and mMBL-C.

Gel-permeation chromatography of mouse serum

Serum from BALB/C mice (100 µl) was subjected to gel-permeation chromatography on a Superose 6 column (30 × 1.0 cm, HR 10/30; Amersham Pharmacia Biotech) equilibrated with running buffer (TBS/Tw containing 2.5 mM EDTA). Chromatography was performed at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. Each fraction was precipitated with 0.6 ml acetone for 2–3 h at −20°C and centrifuged at 10,000 × g for 20 min at 4°C. The pellets were dried in an evaporating centrifuge and redissolved in 25 µl sample buffer for SDS-PAGE and Western blotting. The column was calibrated with the following proteins: thyroglobulin (669 kDa), ferritin (330 kDa; Amersham Pharmacia Biotech), catalase (232 kDa), and HSA (70 kDa). The elution volume of human C1q and IgM was assessed by gel permeation of human serum in the presence of 10 mM EDTA. The content of C1q and IgM in the fractions was measured by enzyme immunosay (EIA).

PAGE

SDS-PAGE was performed in a discontinuous buffer system (18) on 6.5–20% gradient gels. Proteins bands were silver stained as described (19) with the following modifications: Formalin fixation, rinsing with H2O2, and dehydration in acetone were prolonged to 15 min each, and silver impregnation was conducted with a solution of 0.2% (w/v) AgNO3 and 0.25% (v/v) Formalin. Molecular weights were estimated by comparison with prestained marker proteins (Amersham Pharmacia Biotech).

Acid/urea-PAGE was performed as described previously (20, 21) on uniform vertical slab gels (15% (w/v) acrylamide and 0.1% (v/v) bis-acrylamide) in 6.0 M urea and 5.4% (v/v) acetic acid (pH 2.5). The electrophoresis buffer was 5.4% (v/v) acetic acid (pH 2.5). Gels were prerun overnight in electrophoresis buffer at a fixed current of 1 mA. Samples containing 1–2 µg protein were dried in an evaporating centrifuge and redissolved in 15 µl 20 mM sodium borate buffer (pH 9.0) containing 8 M urea and 30 mM DTT. Sample solutions were then boiled for 3 min and acidified with acetic acid to a final concentration of 10% (v/v). Electrophoresis was performed at a fixed current of 9 mA for ~5 h.

Western blotting

Protein bands from PAGE were electroblotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA) (22). Membranes were blocked with TBS containing 0.1% (v/v) Tw for 30 min, cut into strips, incubated with Ab dilution, and developed by the alkaline phosphatase method (23). Strips were washed with colloidal gold to visualize the proteins on the blot (24). For estimating the molecular size of mMBL by gel-permeation chromatography of mouse serum, the Western blots of the fractions were incubated with biotinylated second Ab and developed with enhanced chemiluminescence using HRP-labeled streptavidin (Dako) at 0.2 µg/ml and luminescence reagent (Pierce). Stripping of blots developed by enhanced chemiluminescence was performed by incubation in denaturing and reducing buffer (62.5 mM Tris-HCl, 0.078% (v/v) 2-mercaptoethanol, pH 6.9) at 70°C for 45 min. Blots were then washed overnight in TBS and developed with another Ab.

N-terminal sequencing

The proteins obtained by differential glucose and mannose elution of a mannose-TSK column were subjected to N-terminal amino acid sequencing. Eluate (0.5 ml) containing ~30 µg reduced mMBL was applied to SDS-PAGE. After electrophoresis gels were equilibrated for 10 min in transfer buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid and 10% (v/v) methanol (pH 11)) and the protein bands were transferred to Problot membranes (PE Applied Biosystems, Foster City, CA) at 7.5 volt/cm for 10 h. Protein bands were visualized with Ponceau S, and bands appearing at 28 kDa were cut out and sequenced on an Applied Biosystems 470/120A sequencer. Similarly, the N-terminal sequence of the 21-kDa band appearing in the mMBL-C preparations after the ion-exchange chromatography (see above) was obtained.

Results

Two MBL in mouse serum

Two serum forms of mMBL were found during work to improve the purification of mMBL-A. The eluates obtained from differential monosaccharide elution of a mannos affinity column loaded with the 6.5% PEG 6000 precipitate of mouse serum were analyzed by rocket immunoelectrophoresis (Fig. 1). The MBL in the 10 mM glucose eluate formed rockets of a shape and size consistent with the mMBL-A content found in previous mMBL-A purifications (15). Murine MBL-A eluted over a relatively large number of fractions and the recovery was ~30% of the amount applied to the column as determined by rocket immunoelectrophoresis. When the mannose eluate was analyzed, precipitates of weak intensity and with a pointed shape unlike that of mMBL-A rockets were observed. These pointed rockets varied in character and intensity between different runs and were also occasionally observed when whole mouse serum was analyzed. SDS-PAGE of aliquots from the glucose and mannose eluates showed that the major protein in each eluate had a mobility corresponding to 28 kDa in the reduced state (data not shown). To explain the different appearances on rocket immunoelectrophoresis, the 28-kDa protein bands were blotted onto Problot membranes and subjected to N-terminal sequencing. Analysis of the protein in the glucose eluate yielded the first 25 amino acid residues of mMBL-A, whereas the sequence of the protein in the mannose eluate corresponded to the first 20 amino acid residues of mMBL-C. In both cases, the identity to the published deduced amino acid sequence was 100% and no cross-contamination was observed. Acid/urea-PAGE of the glucose and mannose eluates confirmed the presence of two different forms of mMBL (Fig. 1). The electrophoretic mobilities of the two proteins in this system (mMBL-A > mMBL-C) were consistent with the pIs calculated from their deduced amino acid compositions (7.6 for mMBL-A and 4.8 for mMBL-C).
The specificity of the anti-mMBL-C \(^{9}\) antisera was tested by EIA and Western blotting. EIA showed high Ab binding in wells coated with the synthetic peptide Ag and no binding to wells coated with irrelevant peptides. No specific binding was observed in wells coated with preparations of native mMBL. Western blotting of previously purified mMBL-A (15) and a preparation containing both mMBL-A and mMBL-C (the EDTA eluate of a mannose-TSK column loaded with mouse serum) showed that antisera from the two rabbits recognized mMBL-C but not mMBL-A (Fig. 2).

**Purification of mMBL-A and mMBL-C**

The purification of mMBL-A and mMBL-C was monitored by rocket immunoelectrophoresis against rabbit anti-mMBL antiserum and by Western blotting against biotinylated rabbit anti-mMBL-C \(^{9}\) Ab, respectively. The two proteins were purified by affinity chromatography on a mannose-TSK column eluted with EDTA, removal of Ig by passage through an anti-mouse IgG column, ion-exchange chromatography at pH 6.2 on a Mono-Q column, gel-permeation chromatography on TSK 3000 column, and ion-exchange chromatography on a Resource-Q column. Fractions from the different eluates were analyzed by SDS-PAGE and silver staining (Fig. 3). The two forms of mMBL were well separated by anion-exchange chromatography at pH 6.2. The effluent contained mMBL-A but no mMBL-C, as judged by Western blotting using the biotinylated anti-mMBL-C \(^{9}\) Ab (Fig. 2A), while the bulk of the mMBL-C was eluted with 750 mM NaCl. This eluate contained no mMBL-A when analyzed by rocket immunoelectrophoresis, even when concentrated 10-fold by acetone precipitation. Controls showed nearly 100% recovery of MBL-A after acetone precipitation.

After concentrating mMBL-A on the Resource-Q column, a faint band at 65 kDa was observed in the reduced state (Fig. 3C). This probably represents incompletely reduced mMBL-A, as it bound to the rabbit anti-mMBL-A Ab (results not shown).

**FIGURE 2.** Specificity of the anti-mMBL antisera by Western blotting. A. Reduced mMBL-A, 2 \(\mu\)g. B. Reduced EDTA eluate from mannose-TSK (containing both mMBL-A and mMBL-C). Strips: stained with gold (I), incubated with normal rabbit serum (II), incubated with rabbit anti-mMBL antiserum (III), and incubated with anti-mMBL-C \(^{9}\) antiserum (IV and V) from each of the two rabbits. Bound Ab was detected with alkaline phosphatase-conjugated anti-rabbit IgG.

**FIGURE 3.** SDS-PAGE of fractions from the purification of the mMBLs. A. The EDTA eluate from linked mannose-TSK and antimouse IgG columns. B. The 750 mM NaCl eluate from the Mono-Q column. C. The Resource-Q eluate. D. Void-volume fraction from gel-permeation chromatography. Samples were reduced and gels were silver stained.

**FIGURE 4.** Identification of mMBL-C split product by SDS-PAGE and Western blotting. Strips show reduced 50-\(\mu\)l samples of the 750 mM NaCl eluate from Mono-Q stained with gold (I), incubated with biotinylated rabbit anti-mMBL-C \(^{9}\) Ab (II), and incubated with biotinylated normal rabbit IgG (III). Strips II and III were developed with alkaline phosphatase-conjugated avidin.

**FIGURE 5.** Complement activation. C4 deposition in mannan-coated microtiter wells mediated by mMBL-A, mMBL-C, and human MBL in the presence of \(\text{Ca}^{2+}\) or EDTA. Immobilized C4b was measured by time-resolved fluorometry.
Table I. Monosaccharide inhibition of MBL-binding to mannan

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<thead>
<tr>
<th>Monosaccharide</th>
<th>mMBL-A I₅₀</th>
<th>mMBL-C I₅₀</th>
<th>Human MBL I₅₀</th>
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<td>1-Fuc</td>
<td>1.1 (0.79)</td>
<td>7.8 (3.9)</td>
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<td>αMeMan</td>
<td>1.4 (1.0)</td>
<td>2.0 (1.0)</td>
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<td>GalNAc</td>
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* Concentration (mM) of monosaccharide required for 50% inhibition (I₅₀). I₅₀ values in parentheses are calculated relative to the I₅₀ value of α-methyl-mannose.

SDS-PAGE of the 750 mM NaCl eluate containing mMBL-C (Fig. 3B) revealed the presence of new protein bands between 30 and 40 kDa and a strong band at 21 kDa not present in the EDTA eluate from the mannose-TSK column (Fig. 3A). The N-terminal sequence of the 21-kDa band was identical to that of mMBL-C and the band was stained on Western blots with rabbit anti-mMBL-C Ab (Fig. 4). An aliquot from the 750 mM NaCl eluate was subjected to gel-permeation chromatography on a TSK 3000 SW column. Two major absorbance peaks at 280 nm, of approximately equal size, were observed (data not shown). The first peak emerged with the void volume (>600 kDa), while the second peak emerged at a volume corresponding to 350 kDa. Minor peaks were observed in later fractions. SDS-PAGE of reduced fractions showed the presence of 28-kDa and 21-kDa bands in fractions corresponding to molecular masses from >600 to 230 kDa. In these fractions, the intensity of the 21-kDa band paralleled that of the 28-kDa mMBL-C band. Two fractions in the void volume contained >95% pure mMBL-C, as judged by SDS-PAGE and silver staining, with only trace amounts of other bands at 70 and 100 kDa in the reduced state (Fig. 3D). Fractions from the other major peak at 350 kDa contained bands corresponding to mMBL-C as well as other bands at 40, 70, and 100 kDa under reducing conditions. The >600-kDa peak was used in subsequent analyses for complement activation and carbohydrate-binding specificity.

Approximately 1.6 mg mMBL-A was purified from 135 ml mouse serum as estimated by absorbance at 280 nm. Recovery of mMBL-A in the eluate from the mannose-TSK column was 59% by rocket immunoelectrophoresis, whereas the final recovery was 52%. The protein content in 135 ml serum was measured to 8060 mg (according to the method of Lowry), and the mMBL-A content was measured by rocket immunoelectrophoresis to 3.01 mg. The specific MBL-A content is thus 0.37 μg MBL-A/mg protein. Similarly, the specific MBL-A content of the purified mMBL-A can be calculated to 1.6 mg mMBL-A/mg protein, estimated according to the Lowry method with HSA as standard. The purification factor can thus be calculated at 4251. Based on these recoveries, the concentration of mMBL-A in serum was calculated at ~23 μg/ml. The amount of mMBL-C recovered in the void volume fractions was estimated as 20 μg by absorbance measurements. If all of the 750 mM NaCl eluate had been applied to gel-permeation chromatography, ~0.25 mg mMBL-C would have been recovered in these fractions.

Table II. Monosaccharide specificities of different MBLs

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<th>Human MBL (serum)</th>
<th>Chicken MBL (serum) (26)</th>
<th>Bovine MBL (serum) (27)</th>
<th>Rat MBL-A (serum) (28)</th>
<th>Rat MBL-A (rCRD) (29)</th>
<th>Rat MBL-A (rCRD) (30)</th>
<th>mMBL-A (serum) (15)</th>
<th>mMBL-A (serum)</th>
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<th>mMBL-C (rCRD) (30)</th>
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* Monosaccharide specificities based on 50% inhibition (I₅₀) or Kᵣ values of different MBLs. > denotes more than 10% higher I₅₀ value than the previous monosaccharide, >> denotes more than 50% higher I₅₀ value than the previous monosaccharide, and >>> denotes more than 100% higher I₅₀ value than the previous monosaccharide. Results from the present work are underlined.

FIGURE 6. Oligomerization of MBLs analyzed by Western blotting. Lane I, 0.5 μg mMBL-A, reduced (glucose eluate from mannose-TSK); lane II, the same, unReduced; lane III, mMBL-A and mMBL-C, reduced (10% PEG 6000 precipitate of mouse serum); and lane IV, the same, unReduced. Left panel, incubated with biotinylated anti-mMBL Ab. Right panel, incubated with biotinylated anti-mMBL-C’ Ab. Blots were developed with alkaline phosphatase-conjugated avidin.
Complement activation

The capacity of mMBL-A and mMBL-C to activate complement was determined by measuring C4 deposition in mannan-coated microtiter wells, with human serum depleted of MBL and C1q as the complement source. As shown in Fig. 5, both proteins as well as human MBL mediated C4 deposition, but mMBL-C was less active than mMBL-A and human MBL. Although mMBL-A at 3 ng/ml produced a fluorescence intensity of $10^5$ counts/s, 16 ng mMBL-C/ml was required to produce the same signal. In the presence of EDTA, none of the MBLs produced C4 deposition.

Carbohydrate specificity

The relative potencies of monosaccharides in inhibiting the binding of biotinylated mMBL-A, mMBL-C, and human MBL are given in Table I and compared with previous published observations in Table II. Note the different sensitivity toward inhibition with Glc, αMeGlc, and 1-fuc.

Oligomer state of mMBL-A and mMBL-C

The oligomer state of mMBLs was examined by SDS-PAGE under nonreducing conditions and Western blotting (Fig. 6). The lack of a specific mMBL-A Ab made it necessary to use a preparation of mMBL-A which contained no mMBL-C. The glucose eluate from mannose-TSK was chosen since this preparation had been subjected to the least handling. In parallel, a 10% PEG 6000 precipitate of mouse serum was blotted against rabbit anti-mMBL-C Ab. Both mMBL-A and mMBL-C were observed as a complex mixture of oligomers, varying from dimers of polypeptide chains at 60 kDa to complexes of more than 200 kDa. The pattern was similar for the two types of mMBL. Normal mouse serum was subjected to gel-permeation chromatography and fractions were analyzed by SDS-PAGE and Western blotting. The blots were developed with streptavidin-conjugated HRP followed by enhanced chemiluminescence detection.
(for globular protein markers, Fig. 7a), the first being the main (Fig. 7b). MBL-A also emerged at a position corresponding to a molecular mass of about 600 kDa, but without the second peak observed for mMBL-C (Fig. 7c). Purified mMBL-A was analyzed on the same column. The UV chromatogram and SDS-PAGE of fractions showed that it emerged as a single peak corresponding to an average molecular mass of 600 kDa.

Concentrations of mMBL-A in sera from laboratory strains of mice and wild mouse species and strains were estimated by rocket immunoelectrophoresis in gels containing the anti-mMBL antiserum and with purified mMBL-A as standards (Fig. 8). Serum mMBL concentrations determined by this method varied between

FIGURE 8. Serum mMBL-A concentrations in laboratory strains of mice (A) and wild mice (B), estimated by rocket immunoelectrophoresis.
5 and 50 µg/ml in laboratory strains of mice. The lowest concentration was found in the DW/DW homozygous dwarf strain. Greater variation was found among individuals in wild strains. The least concentration observed in wild mice was 5 µg/ml (Mus musculus domesticus and Mus musculus musculus). The highest concentration of >120 µg/ml was found in a single mouse of the species M. caroli. In general, wild mice showed higher serum mMBL-A concentrations than laboratory strains.

Discussion

Two forms of MBL were identified in mouse serum by their N-terminal amino acid sequences, mobilities on acid/urea-PAGE, and reactivities with an Ab to a synthetic peptide from the deduced amino acid sequence of mMBL-C. Both forms of murine MBL gave bands at 28 kDa on SDS-PAGE in the reduced state.

Two murine serum forms of MBL have been observed before (11). The initial identification of mMBL-C in serum was based on affinity isolation using Ra chemotypes of Salmonella and analysis by acid/urea-PAGE. Two forms of MBL have also been identified in rat serum, where the MBL-C form is described as a minor component compared with MBL-A (10, 31). There has been a tendency in the literature to regard MBL-C as a hepatic protein involved in the intracellular transport of high mannose-type oligosaccharides (32). In contrast to this notion, we find that mMBL-C is a circulating protein present at serum levels comparable to those of mMBL-A. Judged from the recovery and the total yield of the intracellular transport of high mannose-type oligosaccharides in the literature to regard MBL-C as a hepatic protein involved in the transport of mMBL-A purified, an approximate serum concentration of 23 µg/ml was calculated. mMBL-C cannot be quantified, but the experiences gathered suggest a concentration similar to that of MBL-A.

It is possible that MBL-C may differ in abundance and function among different rodent species. Another possibility is that MBL-C often escapes detection because of its sensitivity to proteolytic degradation. Purification of mMBL-C was only possible in the presence of protease inhibitors and preliminary attempts to purify rat MBL-C showed this to be even more susceptible to degradation.

After ion-exchange chromatography at pH 6.2, a 21-kDa fragment of mMBL-C was observed. The relative amount of the 21-kDa fragment increased if purification of mMBL-C was conducted at room temperature or in the absence of protease inhibitors, or when purified mMBL-C was kept at 4°C. The 21-kDa fragment has a N-terminal sequence identical to the terminus of mMBL-C.

Both forms of mMBL mediated C4 activation. mMBL-A was as active as human MBL, whereas mMBL-C was approximately one-fifth as active. Whether the lower activity of mMBL-C reflects an inferior activity in vivo is open to question, as mMBL-C is susceptible to proteolytic degradation and may be split into the 21-kDa fragment during incubation at 37°C, with impairment of complement activation. The finding that mMBL-C activates complement contrasts with the previous observation that MBL-C isolated from rat liver was incapable of complement activation (2). The same has been reported to be the case for human MBL isolated from liver, and also some recombinant human MBL shows a low activating potential (33, 34). The reported lack of complement activation by rat MBL-C and human MBL isolated from liver could be due to incomplete processing with respect to oligomerization and other posttranslational modifications (35). The mMBL-C analyzed for complement activation in this report had an apparent molecular size similar to that of mMBL-A. A similar oligomeric state of mMBL-A and MBL-C was observed by SDS-PAGE under nonreducing conditions followed by Western blotting. Murine MBL-A has previously been observed to emerge from gel-permeation columns at a position corresponding to penta- and hexamers with an average size of 600 kDa, in agreement with the size of rat MBL-A (10, 15). Analysis of mouse serum by gel-permeation chromatography and Western blotting showed that mMBL-C emerged primarily as a peak at ~600 kDa, probably representing hexamers, with a lower amount emerging at ~150 kDa. Purified mMBL-C was found in fractions corresponding to 230–600 kDa on gel-permeation chromatography. MBL-C isolated from rat serum or liver was earlier reported to have an average size of 200 kDa as estimated by gel-permeation chromatography (10, 36). The rat serum MBL-C was purified by a different procedure in which it was separated from MBL-A by gel-permeation chromatography. High-molecular-weight rat MBL-C may have been lost in these experiments and the question of the size of MBL-C in rat serum has so far not been directly addressed. Judged from the complex pattern of oligomers observed by SDS-PAGE, the disulfide bond- ing observed in mMBL-C clearly deviates from the pattern found in recombinant rat MBL-C (37).

Analysis of the carbohydrate specificity showed that human MBL resembles that of mMBL-C more than that of mMBL-A, in agreement with the suggested evolution of MBL genes (38). Likewise, the specificities of mMBLs resemble those of their rat analogues and that of MBL-A concords with previously reported data (Table II) (15). Remarkable is the difference in the specificities of mMBL-A and MBL-C, especially the high affinity of the former for Glc and α-MeGlc. It is likely that the differences in monosaccharide specificity leads to preferential binding to different microorganisms, depending on the composition of glycoconjugates in their outer wall.

Serum concentrations of mMBL-A, estimated by rocket immunoelectrophoresis against MMBL-A standards, were found to range from 5 to 40 µg/ml in laboratory strains of mice and, with a single exception, in wild mice from 5 to 80 µg/ml. The finding of higher mMBL levels in many wild mice is in line with previous measurement (15) in wild yellow-necked mice (M. cervicolor). The variation in serum mMBL in different mice, while significant, is small in comparison with the 500-fold variation (from <10 ng/ml to 5 µg/ml) observed in humans.

The demonstration of both mMBL-A and mMBL-C as serum forms capable of activating the complement system should be remembered when considering animal models for MBL deficiency.

Acknowledgments

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References

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