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Diff erential Expression of the Murine Mannose-Binding Lectins A and C in Lymphoid and Nonlymphoid Organs and Tissues1

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Mannose-binding lectin (MBL)3 is a serum protein that recognizes carbohydrate moieties on the surface of various micro-organisms and activates the complement system in an Ab-independent, calcium-dependent manner (1). Serum MBL forms a complex with the MBL-associated serine proteases MASP-1, -2, and -3 and at least one of these, MASP-2, cleaves the fourth component of complement, C4. C4b, the major cleavage product of C4 binds to and complexes with C2. This C4bC2 complex is substrate of a second MASP-2-mediated cleavage that results in the formation of the classical pathway C3 convertase, C4b2a (2–4).

At the genomic level, MBL has been characterized in many species and has been found in serum in two forms (MBL-A and MBL-C) in rodents, rabbits, and rhesus monkeys, but only in one form in humans and chimpanzees (5). The two murine MBL genes probably arose through gene duplication before human-rodent divergence (6).

In BALB/c mice the serum concentrations of MBL-A and MBL-C were 9.4 and 29 μg/μl, respectively (7). Both types of MBL trigger Ab-independent activation of the complement system (8). Whether they have other common or divergent biological functions is not yet known.

To date, only hepatocytes have been shown to synthesize MBL, except for the rat, in which extrahepatic expression of MBL-A protein has been reported in the kidney (9–11).

Materials and Methods

Mice

BALB/c mice were originally purchased from Charles River Laboratories (Sulzfeld, Germany) and then were maintained and bred under standard conditions in the animal unit of our institute.

Antibodies

Antisera against murine MBL-A and MBL-C were generated by immunizing New Zealand White rabbits (Charles River, Sulzfeld, Germany) twice with 150 μg of purified recombinant murine MBL-A or MBL-C expressed by insect cells (S. Wagner, S. Thiel, F. Petry, and M. Loos, manuscript in preparation). The immunizations were performed in the presence of Ribi Adjuvant (Universal Biologicals, Stroud, U.K.). The specificity of the polyclonal Abs was verified in a Western blot analysis of purified recombinant MBL-A and MBL-C produced in insect cells and by comparison of the staining pattern with that obtained with recently described anti-MBL-A and MBL-C mAbs (7).
### Complementary DNA synthesis

A pool of total RNA was prepared from freshly isolated tissues from six female and four male mice, according to the method of Chomczynski and Sacchi (12). One hundred nanograms of each sample was primed with oligo(dT)23, and single-stranded cDNAs were synthesized using the Superscript First-Strand kit (Invitrogen, Paisley, U.K.) according to the supplier’s instructions.

The integrity of each cDNA sample was checked by PCR using β-actin-specific primers (sense, 5′-TGGAAAACTCTGTCGACCTGAAAC-3′; antisense, 5′-GTGCCAGGCTCTTGTCCTC-3′). The RT-PCR amplification profile consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension for 5 min at 72°C.

### Quantitative real-time RT-PCR

Quantitative PCR was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany), using the FastStart DNA Master SYBR Green I kit (also from Roche), and results were analyzed using the LDCA software supplied with the machine (13). A 295-bp fragment of MBL-A was amplified using primers MBL-A-F1 (5′-CAG GTG GCC GTT GTG GTT-3′) and MBL-A-R1 (5′-TGC TTT CTC TCT AGG TGG-3′). A 305-bp fragment of MBL-C was amplified using primers mC-F1 (5′-GAC CTT AAC CGA AGG GTG TCT AG-3′) and mC-R1 (5′-CAT TTT CTC AGG GCT CTC AG-3′). Each 20-μl PCR contained 1/80th of the original cDNA synthesis reaction (corresponding to 1.25 ng of total RNA), 3.5 mM MgCl2, 0.5 μl of each primer, and 2 μl of the master mix supplied with the kit. Forty-five cycles of amplification were performed: the annealing temperature was reduced from 70 to 58°C during the first 15 cycles and was held constant at 58°C thereafter. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products (14). A standard curve was produced for each form of MBL using serial dilutions of the corresponding cDNAs cloned into pIZ/V5-His (Invitrogen) as templates for the reaction.

### In situ hybridization

MBL-A and MBL-C mRNAs were localized by in situ hybridization using 35S-labeled antisense RNA probes. In vitro transcription and in situ hybridization were performed according to the methods of Melton et al. (15) and Schäfer et al. (16), respectively. The template for the production of the MBL-A probe was a 406-bp fragment of cDNA, stretching from an internal BamHI site to the 3′ end of the cDNA, which was cloned into pBSIISK+.

The MBL-C probe was transcribed from a cDNA template comprising the 264 bp between two internal EcoRI sites, subcloned probe pBSIISK+. To generate specific cRNA probes in antisense orientation, the template was linearized by XhoI digestion, and cRNA was transcribed using T7 RNA polymerase. To control the specificity of hybridization, cRNA probes were transcribed also in sense orientation, linearizing the MBL-A and MBL-C templates by XhoI digestion for in vitro transcription using T3 RNA polymerase.

Twenty-micrometer-thick sections of mouse tissue were cut using a cryostat, mounted on polylysine-coated microscope slides (Merck, Poole, U.K.), and hybridized with the 35S-labeled probes. After hybridization and washing, the signals were detected by exposing the sections to Kodak BioMax MR x-ray film (Sigma-Aldrich, Poole, U.K.) or by dipping the slides in LM-1 photographic emulsion (Amersham Pharma Biotech, Uppsala, Sweden).

### Immunohistochemistry

To locate MBL-A and MBL-C protein expression in different organs by immunohistochemistry, the following procedure was performed. Sections (5 μm) were cut and mounted as described above. Sections were then fixed in PBS/0.4% paraformaldehyde and permeabilized with PBS/0.05% Triton X-100. To avoid nonspecific staining the samples were incubated with PBS/1% H2O2, then blocked with a biotin-blocking system (DAKO, Hamburg, Germany). The sections were incubated with polyclonal rabbit anti-MBL-A or MBL-C Abs (IgG fraction, diluted 1/15). Alternatively, irrelevant rabbit IgG in the same dilution were used for negative controls. All following steps were conducted according to the manufacturer’s instructions with the rabbit ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA). Immunostained sections were counterstained in hematoxylin, analyzed, and photographed with the Zeiss Axioskop 2 and the digital Axiocam HRc (Zeiss, Göttingen, Germany).

### Results

#### Quantification of MBL-A and MBL-C expression in various organs and tissues

The concentrations of MBL-A and MBL-C mRNAs in various mouse tissues were determined by quantitative RT-PCR, using a Roche LightCycler to follow the incorporation of SYBR Green I into the PCR products in real time. A standard curve was prepared for each of the two forms of MBL cDNA using serial dilutions of a plasmid containing the appropriate cDNA as templates for the PCR (Fig. 1A).

Fig. 1B shows the concentration of the MBL mRNAs in 11 different tissues. The liver is the major site of synthesis for both MBL isoforms (69,000 copies/ng RNA for MBL-C vs 23,000 copies/ng RNA for MBL-A; ratio MBL-C/MBL-A = 3). Lower numbers of copies were detected in brain (150 vs 130 copies/ng; ratio = 1), spleen (450 vs 420 copies/ng; ratio = 1), kidney (13 vs 650 copies/ng; ratio = 0.02), and muscle (15 vs 16 copies/ng; ratio ≈ 1). In testis, only MBL-A expression could be detected (57 copies/ng), while in small intestine only MBL-C was found (430 copies/ng). All other tissues contained insignificant (<10 copies/ng) or undetectable amounts.
In situ hybridization was used to localize MBL mRNAs in whole body sections of newborn mice and in sections of adult mouse liver, small intestine, and spleen. Macroscopic examination of the newborn mouse sections and sections of liver from adult mice confirmed that the liver is the principal site of expression for both types of MBL (Fig. 2, A–F), while microscopic examination of liver sections dipped in photo emulsion showed that essentially all hepatocytes express both types of MBL (data not shown). MBL-C expression, but not MBL-A expression, was seen in the small intestine (Fig. 2, G and H). In the spleen a weak, diffuse expression of both MBL mRNAs could be detected throughout the red pulp and in the peripheral macrophage zone (data not shown). The sections were also hybridized with riboprobes transcribed in the sense orientation from MBL-A and MBL-C cDNAs. Within MBL-C sense controls no detectable signals were observed (data not shown).

Those organs where MBL mRNA was detected were analyzed by MBL-A and MBL-C immunohistochemistry (Fig. 3). The results were in good agreement with the in situ hybridization results and demonstrate that wherever MBL mRNA is transcribed, MBL protein is produced. In the spleen MBL-A expression was detected in the peripheral macrophage zone around the germ center (Fig. 3A). MBL-A was also found in the cortical region of the kidney, where strong staining was seen in cells of the glomeruli when observed at higher magnification (×100). In sections of the liver, both MBL-A and MBL-C can be stained throughout the tissue (Fig. 3C). The proteins can be detected in all hepatocytes at comparable strengths. Strong MBL-C protein expression could be seen in the small intestine, especially in the endothelial cells (Fig. 3D).

**Discussion**

The C-type lectin MBL plays an important role in innate immunity by activating the lectin pathway of complement through the MASP-2-mediated cleavage of C4. Two forms of MBL, MBL-A and MBL-C, have been described in rodents (8); it was already known that both are expressed in the liver, and that MBL-A is expressed in the kidney (11). Before both lectins could be separately purified from mouse serum, MBL-C was thought to be a liver protein involved in the transport of oligosaccharides, whereas MBL-A was assumed to be the serum form of MBL (17). This report is the first to describe MBL-A and MBL-C expression in a number of extrahepatic tissues.

Using quantitative real-time PCR, it was found that the liver is indeed the major site of MBL mRNA expression. The level of MBL-C is three times higher than that of MBL-A (69,000 vs 23,000 copies/ng RNA). This ratio is in strikingly close agreement with the ratio of the serum concentrations of MBL-C and MBL-A in BALB/c mice as reported by Liu et al. (7). In situ hybridization
and immunohistochemistry, using MBL-A- and MBL-C-specific cRNA probes and Abs, show that essentially all hepatocytes express both forms of the lectin.

Elsewhere, the highest MBL-A mRNA level was found in kidney, 650 copies/ng RNA. This is ~30-fold less than that in liver, in contrast to previously published results, which indicate that renal MBL-A levels are about one-eighth of those in liver (11). Immunohistochemistry localized the MBL-A-expressing cells to the cortex of the kidney, in particular to the glomeruli. Very little MBL-C could be detected.

Nonhepatic MBL-C expression was highest in the small intestine, where it may play a role in defense against intestinal microorganisms. As proposed for the collectin lung surfactant protein D, which was found to be expressed on several mucosal surfaces in humans and mice, MBL-C as part of the innate immune system could be seen as a counterpart of secretory IgA of the acquired immune system (18, 19). Due to its capability to bind to various microorganisms, MBL-C as well as IgA may protect the small intestine against invading pathogens. In addition, it has been reported that human MBL activates complement after binding to sporozoites of the intestinal parasite Cryptosporidium parvum (20).

In spleen MBL-A and MBL-C mRNA levels were very similar (420 and 450 copies/ng RNA, respectively), and the expression of both types was highest in the marginal macrophage zone. Interestingly, MBL-A and MBL-C were also expressed in the brain (130 and 150 copies/ng RNA, respectively), as is the complement component C1q, which is dramatically up-regulated in response to CNS inflammation (21).

The mRNA levels for MBL-A in testis (57 copies/ng RNA) and muscle (16 copies/ng RNA) and for MBL-C in muscle (15 copies/ng RNA) and kidney (13 copies/ng RNA) are so low that they presumably only play minor roles.

Precisely why rodents have two forms of MBL is not clear. The two lectins have slightly different ligand specificities, which might broaden the spectrum of microorganisms that they can recognize. However, the finding that MBL-A and MBL-C are differentially expressed together with earlier reports that MBL-A, but not MBL-C, is a mild acute phase protein (7) clearly demonstrate that the two genes are independently regulated and imply distinct biological roles for the two lectins.

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References