A Functional Soluble Form of the Murine Mannose Receptor Is Produced by Macrophages in Vitro and Is Present in Mouse Serum*

(Received for publication, May 7, 1998, and in revised form, June 19, 1998)

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A soluble form of the mannose receptor (sMR) has been found in conditioned medium of primary macrophages in vitro and in mouse serum. sMR was released as a single species, had a smaller size than the cell-associated form, and accumulated in macrophage-conditioned medium, in a cytokine-regulated manner, to levels comparable with those found for cell-associated mannose receptor. Pulse-chase experiments showed that sMR production in culture occurred by constitutive cleavage of pre-existing full-length protein. A binding assay was developed to determine the sugar specificity of sMR and its ability to interact with pathogens and particulate antigens (i.e. Candida albicans and zymosan). Protease inhibitor studies suggested that sMR was produced by cleavage of an intact mannose receptor by a matrix metalloprotease or ADAM metalloprotease. A role for sMR in the immune response is proposed based on its binding properties, regulation by cytokines, and the previous discovery of putative ligands for the cytokine-rich domain of the mannose receptor in lymph nodes and spleen.

The mannose receptor (MR)$\dagger$ is a type I integral membrane glycoprotein expressed on many macrophage (MØ) subtypes, monocyte-derived dendritic cells, and hepatic endothelium (1–4). MR is the founding member of a family of molecules sharing the same basic domain structure: an NH2-terminal cysteine-rich domain; a fibronectin type II domain; and a variable number of C-type lectin carbohydrate recognition domains (CRDs), eight in the case of MR, followed by a transmembrane domain and a COOH-terminal intracellular domain (5–7). The other members of the family are: (i) the phospholipase A$_2$ receptor, which contains eight CRDs and is widely distributed (8–10); (ii) the receptor DEC-205, which contains 10 CRDs and is expressed mostly by dendritic cells in T-cell areas of secondary lymphoid organs and by thymic and intestinal epithelia (11–13); and (iii) a recently described molecule found by its homology to the C-type lectin domain of E-selectin (14).

Mannosylated molecules and particles can be endocytosed and phagocytosed through their interaction with the MR (3, 4, 15–17). CRDs, CRD-like domains like the CRDs and the transmembrane and the COOH-terminal intracellular domains. Neither the cytokine-rich domain nor the fibronectin type II domain seems to be required (18, 20). MR synthesis and function are regulated by MØ maturation, lipopolysaccharide (LPS) (3), and cytokines; inhibited by interferon (IFN)-$\gamma$ (6); and up-regulated by IL-4 and -13 (21, 22).

Using as a probe a chimeric protein containing the cytokine-rich domain of the MR fused to the Fc region of human IgG1 (CR-Fc), we found putative ligand(s) for this domain of the MR in marginal zone metallophilic and subcapsular sinus MØ in naïve mice and in the germinal centers of immunized mice. CR-Fc$^\dagger$ cells with dendritic morphology migrated toward the follicular areas and paracortex of lymph nodes during a secondary immune response (23). We hypothesized a role for a soluble form of the MR in the transport of sugar-bearing molecules and/or particles to sites of humoral immune responses. In this report, we demonstrate the existence of such a form in vitro and in vivo. sMR was readily detected in media from cultured primary MØ and was produced by constitutive cleavage of a pre-existing cellular form. A simple and rapid binding assay was developed to test the sugar specificity of sMR (D-mannose = $\alpha$-fucose > $\beta$-galactose) and its ability to interact with particulate antigens (Candida albicans and zymosan) in a carbohydrate-dependent manner. sMR was also detected in mouse serum. Possible functions for sMR will be discussed.

**EXPERIMENTAL PROCEDURES**

Animals—Balb/c and C57BL/6 mice were bred at the Dunn School of Pathology and used at 10–12 weeks of age.

Cytokines, Antibodies, and Reagents—Murine IL-4 was from R&D Systems (Minneapolis, MN). Murine IFN-$\gamma$ was from Genzyme Diagnostics (Cambridge, MA). Rabbit polyclonal antibody against purified murine mannose receptor (24) was absorbed with thymocytes. Rabbit polyclonal antibody against the COOH end of the MR was obtained by immunizing New Zealand White rabbits with the synthetic peptide NH$_2$-SNLSPGTSDTKDLGNIQNEH-COOH (200 $\mu$g of peptide per immunization). LPS, zymosan from Saccharomyces cerevisiae, $\alpha$-mannose, $\alpha$-fucose, $\beta$-galactose, mannose, p-aminophenyl-$\alpha$-mannosyl-agarose, and p-aminophenyl-$\alpha$-$\beta$-galactosyl-agarose were purchased from Sigma (Poole, Dorset, UK). Mannose-BSA (Man$_2$-BSA, 32 mol of mannosyl/mol of bovine serum albumin) was purchased from EY Laboratorios (San Mateo, CA). The protease inhibitors N-(trans-carboxy-2-carbonyl)-i-leucyl-agmatine (E-64, cysteine proteinase inhibitor), pepstatin (acid proteinase inhibitor), and a $\alpha$-macroglobulin (host defense proteinase inhibitor) were from Boehringer Mannheim (Lewes, UK). The matrix metalloprotease inhibitor BB 2116 was a kind gift of Dr. Andrew Gearing of British Biotech (Oxford, UK). Peroxidase-conjugated donkey anti-rabbit IgG was from Chemicon (Harrow, UK). Protein G-Sepharose was from Amersham Pharmacia Biotech (Upp-
Supernatants were cleared in the same way. 100 mM Tris-HCl, pH 8, 500 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% IL-4 or 10–50 μM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 1 mM NaCl, 0.05% (w/v) SDS; and once in 10 mM Tris-HCl, pH 8, 0.05% (w/v) SDS; proteins were eluted in nonreducing loading buffer, resolved by SDS-PAGE, and visualized by fluorography. In some cases, proteins were eluted by incubating the beads with nonreducing loading buffer and centrifugation and washed 3 times with binding buffer. Bound proteins were detected under these conditions (data not shown). sMR was not detectable in cell lysates or supernatants from Bio-Gel-elicited peritoneal MØs. sMR synthesis was regulated by MØ maturation, LPS (3), and cytokines. It is inhibited by EDTA. Binding of sMR to the beads was inhibited by millimolar concentrations of D-mannose and L-fucose but not by D-galactose. sMR was detected after 24 h of culture and accumulated in the conditioned media. Only one form of sMR with slightly faster mobility than cMR was found. No release of another MØ-specific integral membrane protein, F4/80, was detected under these conditions (data not shown). sMR was not recognized by an antibody specific for the cytoplasmic tail of the MR (Fig. 2). This result suggests that sMR lacks this domain and could be produced by proteolytic cleavage of the cell-associated form of the receptor. sMR was stable, was not affected by freezing and thawing, and could be stored at −20 °C for long periods of time.

**sMR Ligand Binding Properties**—The binding properties of sMR were characterized using a semiquantitative ligand binding assay. sMR bound specifically to p-aminophenol p-D-mannose-agarose, and this interaction was inhibited by EDTA. sMR was detected under these conditions (data not shown). sMR was not detectable in cell lysates or supernatants from Bio-Gel-elicited peritoneal MØs. sMR synthesis was regulated by MØ maturation, LPS (3), and cytokines. It is inhibited by EDTA. Binding of sMR to the beads was inhibited by millimolar concentrations of D-mannose and L-fucose but not by D-galactose (Fig. 3), in good agreement with the published specificity for MR: D-mannose ≈ L-fucose > D-galactose. As expected, mannann and mannann-BSA were much more potent inhibitors of sMR binding, with approximate IC_{50} values of 10 nM and 3 μg/ml, respectively (not shown). The same type of assay was used to test the ability of sMR to bind natural particulate antigens, i.e., heat-killed C. albicans or zymosan (Fig. 4). In both cases the interaction was inhibited by D-mannose and L-fucose (10 mM) but not by 10 mM D-galactose.

**Regulation of sMR Production**—MR synthesis is regulated by MØ maturation, LPS (3), and cytokines. It is inhibited by IFN-γ (6) and up-regulated by IL-4 and -13 (21, 22). To characterize in more detail the relationship between cMR and sMR production, pulse-chase experiments were performed under different culture conditions, as described under “Experimental Procedures.” Fig. 5 shows the effect of IL-4, IFN-γ, and LPS on cMR and sMR synthesis. After the pulse, two bands corresponding to the precursor and mature forms of cMR (3) were

**FIG. 1. A soluble form of the MR detected in supernatants from Bio-Gel-elicited peritoneal MØ.** Western blot analysis of cell lysates (15 μg, upper panel) and conditioned media (lower panel) from Bio-Gel-elicited MØ cultured in the absence or presence of FCS and/or IL-4 and collected at days 0 (d0), 1 (d1), and 3 (d3) with a polyclonal antibody against murine MR is shown. The asterisk indicates cultures in which medium was changed on day 1.
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Fig. 2. An antibody specific for the cytoplasmic tail of the MR does not recognize sMR. Cell lysate (7 μg, C) and concentrated supernatant (1, 2.5, and 5 μL, S) from Bio-Gel-elicted MØ cultured in RPMI-2% FCS in the presence of IL-4 (10 ng/ml) were analyzed by Western blot with polyclonal antibodies raised against purified murine MR (α-MR) or a synthetic peptide with part of the COOH-terminal sequence of murine MR, as described under "Experimental Procedures." The control panel shows results obtained with preimmune antibody. α-MR-COOH failed to recognize the soluble form of the MR found in the supernatant.

![Image](57x475 to 289x566)

**Fig. 3.** Carbohydrate binding activity of sMR. Concentrated supernatant from MØ cultured in the presence of 2% FCS and IL-4 was incubated with p-amino phenyl α-D-mannosyl-agarose in the absence (control) or presence of EDTA (10 mM), D-mannose, L-fucose, or D-galactose (0.01, 0.1, 1, or 10 mM), as described under "Experimental Procedures." Bound material was analyzed by Western blot using the anti-MR polyclonal antibody. sMR bound specifically to mannosylated agarose beads in a Ca²⁺-dependent manner, and this interaction could be inhibited by D-mannose and L-fucose but not by D-galactose.

![Image](308x626 to 554x729)

**Fig. 4.** Binding of sMR to heat-killed Candida albicans and zymosan. Concentrated supernatant from MØ cultured in the presence of 2% FCS and IL-4 was incubated with p-amino phenyl α-D-mannosyl-agarose (Man-O), 2 × 10⁵ zymosan particles, or 2 × 10⁵ heat-killed C. albicans in the absence (−) or presence of D-mannose (10 mM), L-fucose (10 mM), or D-galactose (10 mM), as described under "Experimental Procedures." Bound material was analyzed by Western blot using the anti-MR polyclonal antibody. sMR bound to heat-killed C. albicans and zymosan in a Ca²⁺- and mannose and fucose-dependent manner.

recognized by the anti-MR polyclonal antibody in cell lysates. cMR synthesis was increased by IL-4 and decreased by LPS. No obvious difference in the rate of synthesis was observed in the case of IFN-γ under these conditions. sMR could already be detected in supernatants collected after a 2-h pulse in samples treated with IL-4. Analysis of samples collected after the 3-h chase showed the disappearance of the precursor form in the cell lysates (probably chased into the mature form of cMR) and the presence of sMR in the conditioned media from IL-4-treated cells. IFN-γ and LPS treatments seemed to reduce the half-life of the cellular receptor, although the reduction in the intensity of the bands could be because of the production of sMR, which is undetectable at this time point. After the 8-h chase, sMR could be detected in supernatants from control and IL-4- and IFN-γ-treated cells in amounts proportional to their rate of synthesis. This finding indicated that the production of sMR was constitutive and that the amount of sMR correlated with the amount of cMR present in the cell. No differences in sMR production were observed when IL-4 or IFN-γ was added only during the chase period (data not shown).

**sMR Production Is Blocked by an Inhibitor of Matrix Metalloproteinases**—As a first step toward characterization of the cleavage event that generates sMR, peritoneal MØs were pulsed with Tran35S-label followed by a chase in the presence of a variety of protease inhibitors. As shown in Fig. 6, the protease inhibitor BB 2116, a synthetic hydroxamic acid-based inhibitor of matrix metalloproteinases (26, 27), blocked production of sMR in a concentration-dependent manner, with an IC₅₀ of approximately 3 μg/ml. The decrease in sMR production was unlikely to be caused by cell toxicity, because even at the highest concentration tested, 50 μg/ml, cell viability was greater than 95% by trypan blue staining. Other protease inhibitors tested were either ineffective or had modest and/or variable effects (Fig. 6).

**sMR Is Present in Mouse Serum**—The detection of sMR in conditioned media from cultured peritoneal MØ might be considered an artifact of this *in vitro* system. For that reason, the presence of sMR in normal mouse serum was examined. A protein that reacted with the anti-MR polyclonal antibody and comigrated with sMR from MØ-conditioned media could be detected using just 0.5 μL of mouse serum (data not shown). This protein displayed sugar binding activity similar to the activity described above for sMR (Fig. 7). These findings indicate that sMR production occurs *in vivo* and that sMR is a component of normal mouse serum. sMR levels in mouse serum were not affected by *Mycobacterium bovis* bacillus Calmette-Guérin or *C. albicans* infection, or by intraperitoneal treatment with LPS, zymosan, or heat-killed *C. albicans* (data not shown).

**DISCUSSION**

In this study we demonstrate that there is a functional soluble form of murine MR produced by MØ *in vitro* and present in mouse serum. sMR migrated faster than cMR on SDS-PAGE, presumably because of cleavage of the transmembrane domain and cytoplasmic tail (see Fig. 2), and was produced by cleavage of a pre-existing full-size cMR (Fig. 5). No sMR was found in cell lysates. sMR could be stored at −20 °C for long periods of time with no obvious change in mobility on SDS-PAGE or in sugar binding activity. The production of a single species of sMR is consistent with specific cleavage of cMR to produce a functional soluble form. sMR was able to interact with carbohydrates in a mannose- and fucose-dependent manner (Figs. 3 and 4). These results agreed with previous studies...
of sugar specificity of purified human MR (15) or complete and truncated forms of the human receptor expressed in vitro (18). The assays developed here allow the direct study of sMR interactions with a wide range of pathogens in a cell-free system. We showed that sMR interacted with heat-killed C. albicans and zymosan (prepared from the S. cerevisiae cell wall) in the same way as it bound p-aminophenyl α-D-mannosyl-agarose. Phagocytosis of unopsonized C. albicans by MØ has been shown to be mediated, to a considerable extent, by the MR; mannan and mannose-BSA inhibited 80% of C. albicans uptake by human monocyte-derived MØ (25) and murine peritoneal MØ.2

The inhibition of sMR production by the matrix metalloprotease inhibitor BB 2116 strongly suggests a role for a matrix metalloprotease in the release of sMR from the membrane. The matrix metalloproteases are a large family of extracellular zinc proteases, at least 18 distinct members of which have been discovered (28). A growing number of integral membrane proteins in the immune system have been shown to be released from cells by the action of metalloproteases, including tumor necrosis factor α (TNFα) (26, 29), TNFα receptor (30), L-selectin (31), and Fas ligand (32). TNFα, perhaps the most well understood of this group, is released from MØ by the action of TNFα-converting enzyme (33, 34) and/or ADAM10 (35, 36), both members of the ADAM family of metalloproteases. At least 17 members of this family, which feature metalloprotease and disintegrin domains, have now been identified (28). Because BB 2116 can also inhibit TNFα release (26), the sMR releasing activity may be either a matrix metalloprotease or an ADAM family metalloprotease. Further studies with specific inhibitors should help to answer this question.

Unlike cMR synthesis, sMR production per se was not regulated by cytokines (IFN-γ and IL-4), but because the amount of sMR in conditioned media of cytokine-treated cells was directly related to the amount of cMR present in the cell, the total amount of sMR was effectively cytokine-regulated and followed the pattern described for cMR (6, 22). It should be noted that IFN-γ (Fig. 5) and phorbol 12-myristate 13-acetate-induced down-regulation of MR (data not shown) could not be explained by increased cMR processing. Therefore, at least in these cases, sMR production was not a mechanism of down-modulation of MR activity as described for other leukocyte surface molecules (37, 38).

One of the best characterized lectins in serum is the mannos binding protein (MBP) (39), a C-type lectin with specificity for mannos and N-acetylgalcosamine. Major differences between the MBP and sMR are suggestive of complementary roles in their interaction with mannosylated antigens. (i) In the case of MBP, 32-kDa monomers, each containing one CRD, form clusters in serum, but they would not be necessary for its binding to glycoconjugates. (ii) MBP can activate the classical pathway of complement and act as an opsonin; MBP-pathogen complexes could be cleared through the interaction of the collagen region of MBP with collectin receptors expressed by most phagocytic cells. Ligand(s) for the cysteine-rich domain of the MR has been found on marginal zone metallophilic MØ in spleen, subcapsular sinus MØ in lymph nodes, germinal centers (specific cell type unknown), and a population of cells with dendritic morphology that migrate into the follicles during an immune response...
(CR-Fc⁺ cells) (23). sMR could interact with whole infectious agents (i.e. C. albicans) or cleavage products released after phagocytosis through the CRDs and with ligand(s) present on the cells described previously through the cysteine-rich domain. In this way, sMR-bound antigens would not be targeted for clearance but delivered, in their native form, to the CR-Fc⁺ cells surrounding the white pulp of the spleen or follicular regions in lymph nodes. Accordingly, sMR levels were increased in the presence of IL-4, a Th2 cytokine. We propose that mannosylated antigen opsonized by the MBP could also be targeted by this receptor.

Recently, a liver-specific form of the MR was identified as responsible for the clearance of the glycoprotein hormone lutropin through the recognition of the sulfated carbohydrate structure SO₄−4-GalNAcβ1,4Glc-NAcβ1,2Manα (42, 43). The binding site has been localized to the cysteine-rich domain of the receptor (44). Therefore, the possible competition of lutropin with the cysteine-rich domain ligands found in lymphoid organs should be considered. The tissue specificity of this recognition and the heterogeneity in binding properties found for recombinant MR expressed in Chinese hamster ovary cells (43) suggest that post-translational modifications could alter the binding properties of the cysteine-rich domain and determine the ability of MR to bind lutropin. In the case of sMR, no data are yet available regarding its cellular origin in vivo (e.g. hepatic endothelium versus MØ) or its ability to interact with lutropin.

The MR has been implicated in the uptake of mannosylated antigens and their delivery to different intracellular compartments for their presentation in association with major histocompatibility complex class II in the case of peptides (4) or CD1 in the case of nonpeptides (16). We propose a possible third mechanism of presentation of glycoconjugates: in their native form bound to sMR and attached to the cysteine-rich domain ligand(s) expressed by specialized antigen-bearing cells.

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