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Superoxide production from human polymorphonuclear leukocytes by human mannan-binding protein (MBP)

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Mannan-binding protein (MBP) is a Ca^{2+}-dependent mammalian lectin that plays an important role in innate immunity. In this study, we found that ligand-bound MBP stimulates polymorphonuclear leukocytes (PMN) to induce cell aggregation and superoxide production. The biological response of PMN to ligand-bound MBP was dose- and time-dependent. The PMN aggregation and superoxide production induced by ligand-bound MBP was blocked completely by pertussis toxin, and partially blocked by a platelet activation factor receptor antagonist, TCV-309. These findings suggest that the ligand-bound MBP stimulates PMN through a putative MBP receptor(s) on PMN.
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Introduction

Mannan-binding protein (MBP), also called mannose-binding protein (MBP) or mannan-binding lectin (MBL), is a Ca^{2+}- dependent (*C*-type) serum lectin specific for mannose, *N*-acetylglucosamine and fucose [1–3]. Human MBP is a homooligomer of an approximately 31 kDa subunit, each subunit containing a carbohydrate recognition domain (CRD) followed by a short neck region at the COOH terminal side and a collagen-like domain followed by a short cysteine-rich region at the NH_2 terminal side. Three subunits form a structural subunit and MBP normally consists of 2–6 structural subunits joined through disulfide bonds at the *N*-terminus, the whole molecular mass being approximately 200–600 kDa [4]. MBP is an important factor in innate immunity especially against microbial infections. Thus, MBP triggers carbohydrate-mediated complement activation through a lectin pathway [5], kills bacteria with the help of complement [6], functions as a direct opsonin [7] and prevents virus infection [8]. Structural variants as to the collagen-like domain are known to be associated with a low serum concentration of MBP and an opsonic defect [9,10].

Several years ago, we found that some mammalian cells including certain cancer cell lines express the carbohydrate ligand for MBP [11]. In addition, the *in vivo* growth of one of these cell lines, SW1116, a colorectal cancer cell line, was shown to be suppressed dramatically by the administration of recombinant vaccinia virus carrying the human MBP gene [12]. We proposed to term this novel activity "MBP-dependent cell-mediated cytotoxicity". In this study, we have tested the response of polymorphonuclear leukocytes (PMN) to MBP immobilized on synthetic carbohydrate ligand coated wells as a model for tumor cell surfaces that express the MBP ligands at high level.

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Materials and methods

Materials

Nitroblue tetrazolium, superoxide dismutase and retinoic acid were obtained from Sigma Chemical (St. Louis, MO, USA). A platelet activation factor receptor antagonist, TCV-309, was a kind gift from Takeda Chemical Industry, Co., Ltd. (Osaka, Japan). Poly [N-*p*-vinylbenzyl-O-ß-D-mannopyranosyl-

[1,4]-D-mannamide] (PV-Man) and pertussis toxin were obtained from Seikagaku-kogyo (Tokyo, Japan). RPMI 1640, Dulbecco's modified Eagle medium (DMEM) and Hanks balanced salt solution (HBSS) were obtained from Nissui (Tokyo, Japan). Fetal calf serum was obtained from GIBCO (Grand Island, NY). Alkaline phosphatase-conjugated anti mouse IgG was obtained from Vector (Burlingame, CA). The human hepatoma cell line, HLF (JCRB 0405) was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). HLF cells were cultured in DMEM supplemented with 10% FCS. Wild type and G54D mutant recombinant human MBP were prepared as described previously [13]. The purified recombinant human MBP gave a single band corresponding to 32 kDa on SDS-PAGE under reducing conditions (data not

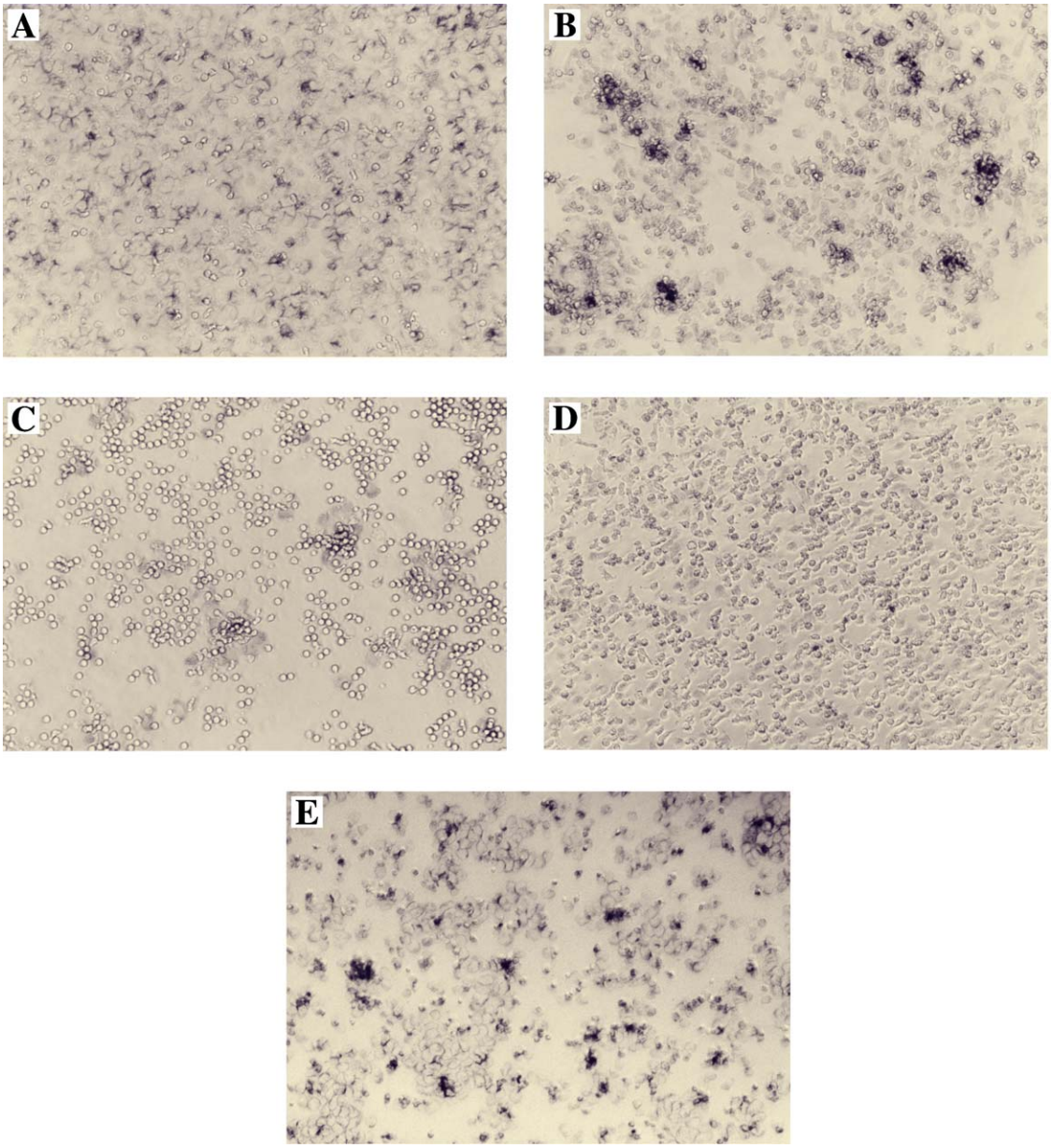

Figure 1. Stimulation of PMN aggregation and superoxide production by MBP bound to ligand-coated plates. The wells of 96-well tissue culture plates were coated with PV-Man (A–E). After coating with PV-Man, the wells were subsequently coated with 50 μl of recombinant human MBP (B–D) (50 μg/ml) or recombinant human G54D mutant MBP (50 μg/ml) (E). The subsequent coating with MBP was omitted for an negative control experiment (A). Isolated PMN (1.5×10^5 cells) were incubated with 100 μg/ml NBT for 2 h in the wells (A–E). For inhibition experiments, PMN were incubated with ligand-bound MBP in the presence of 50 μg/ml SOD (C) or preincubated with 1 μg/ml PT prior to the incubation with ligand-bound MBP (D). The cells were observed under a phase-contrast microscope and photographed. Original magnification, $\times$200.

shown). Blood was obtained from healthy volunteers. PMN were isolated using Polymorphoprep, obtained from Nycomed Pharma AS (Oslo, Norway), according to the manufacturer's instructions.

Quantification of MBP bound to PV-Man coated plates

The amount of MBP bound to PV-Man-coated wells was determined by ELISA using an anti-human MBP monoclonal antibody as follows. Microtiter plates (FALCON 353072. Becton Dickinson, Lincoln, NJ), were coated with PV-Man at 100 μg/ml in H_2O (50 μl/well) for 18 h at 4°C, and then blocked with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 3% BSA and 0.1% NaN_3 for 2 h at room temperature. After washing with TBS containing 0.05% Tween 20 and 10 mM $CaCl_2$ (TBS-Tween-Ca), the wells were incubated for 2 h at room temperature with a sample containing MBP (50 μl/well), which was diluted with TBS containing 1% BSA, 10 mM $CaCl_2$ and 0.1% NaN_3 (TBS-BSA-Ca-NaN_3). After washing with TBS-Tween-Ca, 10 μg/ml of a monoclonal antibody to human MBP (YM304) in TBS-BSA-Ca-NaN_3 (50 μl/well) was added to the wells. After incubation for 18 h at 4°C and washing with TBS-Tween-Ca, the wells were incubated with alkaline phosphatase-conjugated anti mouse IgG diluted 1000-fold in TBS-BSA-Ca-NaN_3 (50 μl/well) for 2 h at room temperature and then washed with TBS containing 10 mM $CaCl_2$. *p*-Nitrophenylphosphate at the concentration of 1 mg/ml in 50 mM Na_2CO_3 containing 0.5 mM $MgCl_2$ was added to each well (100 μl/well). After incubation at room temperature for 30–90 min, the OD_{405} of each well was measured using a microplate reader (BioRad). Purified recombinant human MBP was used as a standard. The amount of MBP bound to the surface of the plastic plates increased linealy from 10 to 200 ng MBP/ml.

Detection of superoxide production by PMN

The superoxide anion was detected as the reduction of nitroblue tetrazolium (NBT). The PMN suspension (50 μl, 3×10^6 cells/ml) containing NBT in HBSS (100 μg/ml) was plated on PV-Man- and MBP-coated wells, followed by incubation at 37°C for 2 h unless otherwise stated. Black-blue formazan deposits formed on NBT reduction were observed under a phase contrast microscope (Nikon, Tokyo, Japan). To confirm that the reduction of NBT was caused by the superoxide anion, superoxide dismutase was added to the PMN suspension at the final concentration of 50 μg/ml prior to plating to the plate. The effects of pertussis toxin (PT) and a PAF receptor antagonist, TCV-309, on the PMN stimulation by MBP were examined by preincubation of PMN with PT at 1 μg/ml at 37°C for 90 min or TCV-309 at various concentrations in the range of 50–1000 nM at 37°C for 30 min prior to plating on MBP-coated wells.

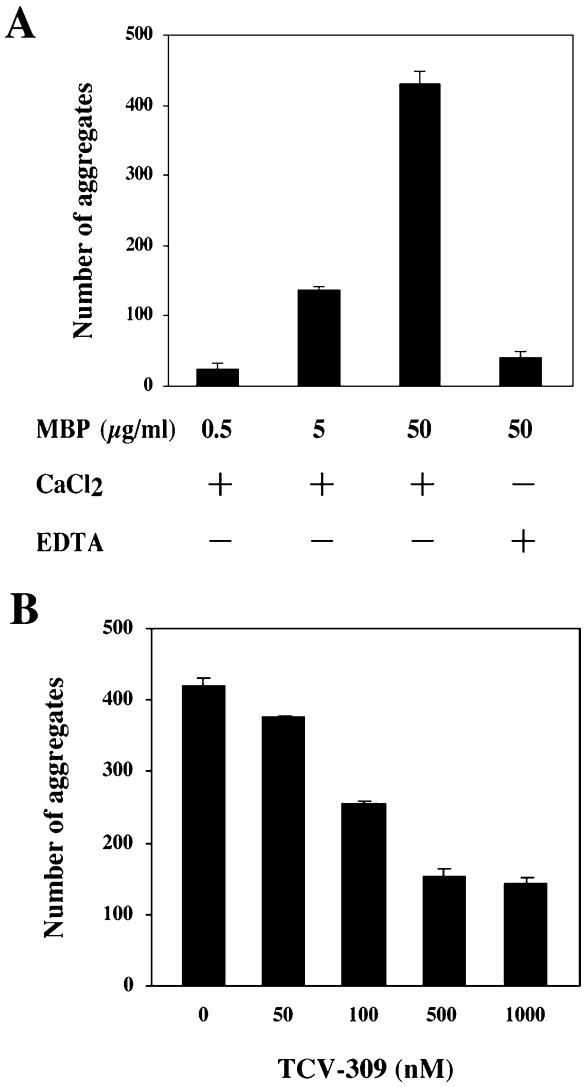

Figure 2. (A) Dose dependency of PMN aggregation and superoxide production induced by ligand-bound MBP. Wells were coated with PV-Man, followed by subsequent coating with different concentrations of recombinant human MBP, as indicated. Negative controls were coated with 50 μl of recombinant human MBP (50 μg/ml) in the presence of 4 mM EDTA. Isolated PMN were incubated with 100 μg/ml NBT for 2 h in those wells and observed under a phase-contrast microscope. The numbers of aggregates accompanying by formazan deposits in the wells were determined. The results are presented as means + SD for two experiments. (B) Effects of a PAF receptor antagonist, TCV-309, on PMN aggregation and superoxide production induced by ligand-bound MBP. Wells were coated with PV-Man, followed by subsequent coating with 50 μl of recombinant human MBP (50 μg/ml). Isolated PMN were added to the wells and then incubated with 100 μg/ml NBT for 2 h in the presence of different concentrations of TCV-309. The numbers of aggregates with blue-black granules in the wells were determined. The results are presented as means + SD for two experiments.

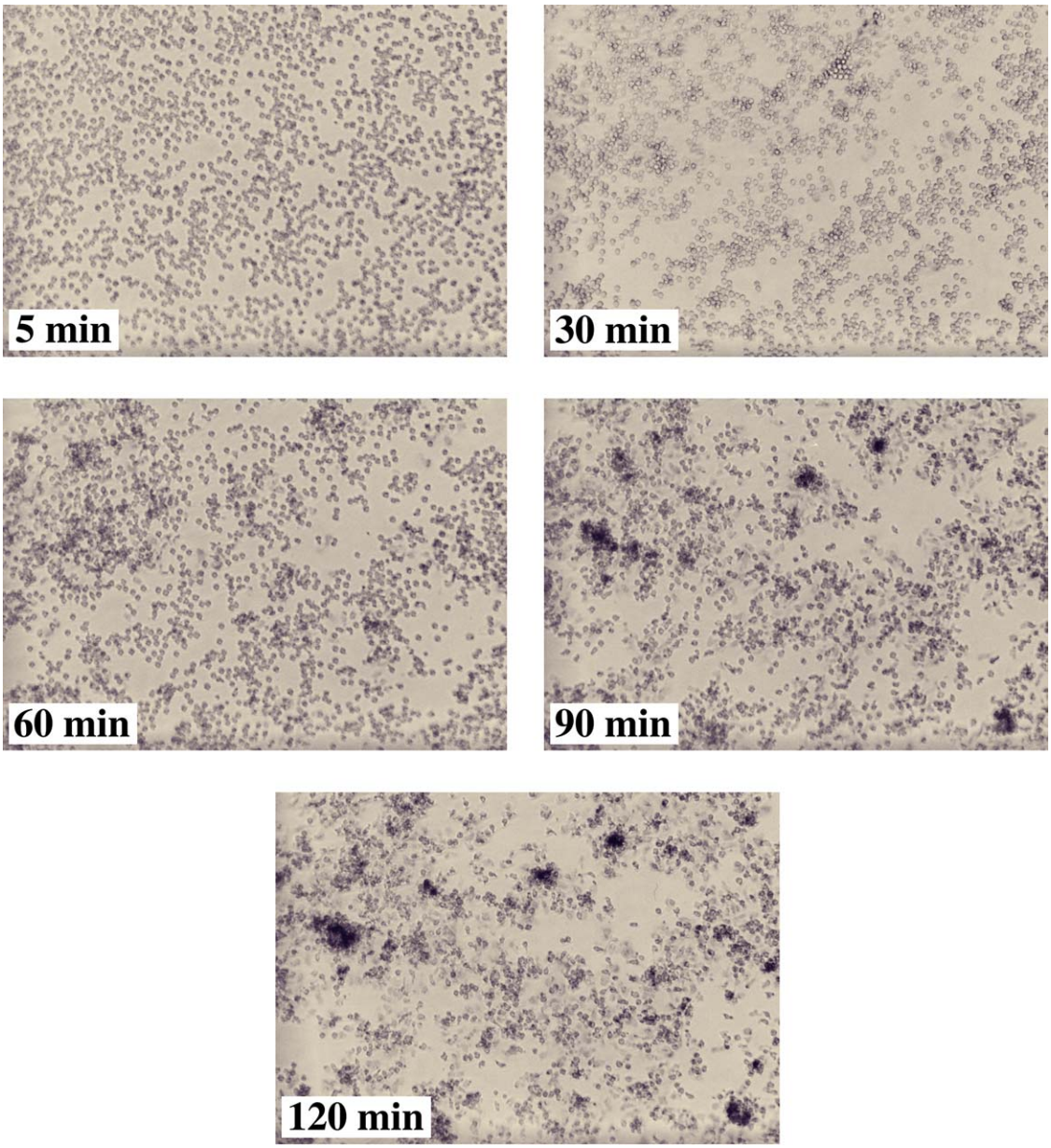

Figure 3. Time courses of PMN aggregation and superoxide production induced by ligand-bound MBP. Wells were coated with PV-Man, followed by subsequent coating with 50 μl of recombinant human MBP (50 μg/ml). Isolated PMN were incubated with 100 μg/ml NBT in the wells. During the incubation, the same field was observed under a phase-contrast microscope and photographed at a different elapsed time, as indicated. Original magnification, $\times$200.

Results and discussion

Ligand-bound MBP induces PMN aggregation and superoxide production by PMN

To determine whether or not ligand-bound MBP induces the activation of PMN, we used microtiter culture plates containing ligand-bound MBP.

To exclude the effects of serum proteins other than MBP, we used highly purified recombinant human MBP. After 2 h incubation, aggregates of PMN accompanying by formazan deposits were observed in the wells containing ligand-bound MBP (Figure 1B). In contrast, the wells coated with PV-Man alone did not show any significant formation of formazan deposits (Figure 1A). Formazan deposits were not induced in the presence of superoxide dismutase (Figure 1C), indicating that the formazan deposits were generated by the superoxide anion. As shown in Figure 2A, the number of PMN aggregates accompanying by formazan deposits was dependent on the amount of MBP added. Furthermore, when MBP had been incubated on PV-Man-coated wells in the presence of EDTA,

the number of formazan deposits was greatly reduced, in agreement with the requirement of Ca^{2+} for MBP-ligand binding (Figure 2A).

Then, we examined whether or not the G54D mutant MBP, has the ability to induce the activation of PMN. The G54D mutant is one of the allelic forms of human MBP, in which glycine is substituted by aspartic acid at codon 54 in the fifth collagen repeat, and lacks complement-activating activity [14,15]. This mutation is associated with an increased frequency of recurrent infection in infants and adults [11,16]. As shown in Figure 1E, the G54D mutant recombinant MBP also stimulated PMN to induce aggregation and NTB reduction, indicating that the response of PMN by ligand-bound MBP is complement-independent. These results are similar to the anti-tumor activity of MBP *in vivo* in terms of an overlapping function of the wild type and the G54D mutant MBP.

Figure 3 shows the time-course of PMN aggregation and superoxide production induced by ligand-bound MBP. The aggregation of PMN was observed after as early as 30 min incubation with ligand-bound MBP. Formazan deposits in PMN aggregates were demonstrable after 90 min stimulation. These results indicate that ligand-bound MBP stimulates PMN to induce aggregation at first, and then super oxide production afterwards, suggesting that the activation of PMN by ligand-bound MBP is mediated by certain factors which induce aggregation and super oxide production of PMN.

The number of PMN aggregates accompanied by formazan deposits was dependent on the amount of MBP added (Figure 2A), while comparable amounts of soluble MBP did not induce PMN aggregation and superoxide production (data not shown). These results suggest that stimulation of PMN by MBP requires a certain density of ligand-bound MBP for a putative MBP receptor expressed on PMN. It should be noted that there is a previous report indicating that MBP did not stimulate superoxide production by PMN [17]. However, in these experiments, MBP was directly coated onto the wells of polystyrene microtiter plates and might bind to the surface in a random orientation, probably leading to attenuation of the density of the effecter sites of MBP for the stimulation of PMN.

Effects of PT and a PAF antagonist on PMN aggregation and NBT reduction induced by ligand-bound MBP

A number of stimuli that activate PMN are known to be sensitive to pertussis toxin (PT) (*e.g.* fMLP, C5a, leukotriene B4, and platelet-activating factor (PAF)) [18]. PT catalyzes the ADP ribosylation of certain G protein α-subunits, resulting in the uncoupling of PT-sensitive G proteins and cell surface receptors [19]. As shown in Figure 1D, the aggregation and superoxide production by PMN induced by ligand-bound MBP were completely inhibited by PT treatment, suggesting that a *G*-protein coupled receptor is associated with the stimulation of PMN by ligand-bound MBP.

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) induces superoxide production by neutrophils and eosinophils [20]. To study the possibility that PAF is involved in the activation of PMN by ligand-bound MBP, we examined the effect of TCV-309, a potent and highly specific antagonist of PAF [21]. As shown in Figure 2B, TCV-309 inhibited PMN aggregation and superoxide production induced by ligand-bound MBP in a concentration-dependent manner. At the concentration of 1000 nM, TCV-309 inhibited the PMN response to ligand-bound MBP by 66%. These results may indicate that PMN aggregation and superoxide production induced by ligand-bound MBP are mediated in part by endogenously generated PAF.

With regard to a putative receptor for MBP, two C1q receptors, C1qRp and calreticulin (cC1qR) [22,23], and complement receptor 1 (CR1) have been shown to interact with MBP [24]. However, it is clear that further work is required to characterize the putative MBP receptor that mediates PMN aggregation and superoxide production.

In summary, we have demonstrated that the ligand-bound MBP induces PMN aggregation and superoxide production by PMN without the help of complement component. Since PMN has been shown to suppress certain tumors in response to cytokines [25], it may be possible that PMN stimulated by ligand-bound MBP is associated with the tumor suppression by MBP.

Acknowledgments

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