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Complement-dependent cytotoxic activity of serum mannan-binding protein towards mammalian cells with surface-exposed high-mannose type glycans*

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Serum mannan-binding protein (S-MBP), a lectin specific for mannose and *N*-acetylglucosamine, activates complement through the classical pathway. With the help of complement, S-MBP lyses red blood cells which have been coated with yeast mannan and kills bacteria which have *N*-acetylglucosamine and/or L-glycero-D-manno-heptose on their core oligosaccharide. In this study, we examined whether mammalian cells, on which S-MBP could bound, are killed by a complement-dependent mechanism. When baby hamster kidney (BHK) cells were treated with an α-mannosidase inhibitor, 1-deoxymannojirimycin (dMM), most of the cellular oligosaccharides were transformed from the complex-type to the high mannose-type. S-MBP bound to the dMM-treated BHK cells in the presence of Ca^{2+}, and this binding was eliminated by mannose. When dMM-treated cells, labelled with ^{51}Cr, were incubated with complement, radioactivity was released in a dose-dependent manner by S-MBP and complement. This release was not observed with heat-inactivated complement. These observations suggest that S-MBP is able, with the help of complement, to kill not only exogenous microorganisms but also mammalian cells which have high mannose-type oligosaccharides exposed on their surfaces.

Keywords: serum lectin; complement activation; cytotoxicity; 1-deoxymannojirimycin

Abbreviations: S-MBP, serum mannan-binding protein; BHK, baby hamster kidney; dMM, 1-deoxymannojirimycin; FITC, fluorescein-isothiocyanate; TBS, Tris buffered saline; HIV, human immunodeficiency virus; CH_{50}, 50% haemolytic units. The complement nomenclature used was recommended by the World Health Organization (1986).

Introduction

The serum mannan-binding protein (S-MBP), a lectin recognizing mannose and *N*-acetylglucosamine, has been isolated and characterized from various mammalian sera [1–5]. S-MBP is a macromolecule with an apparent molecular mass of about 650 kDa, consisting of approximately 18 identical subunits of around 31 kDa. The subunit has a carbohydrate recognizing domain on its COOH-terminal and a collagen-like domain consisting of 18–20 repeats of the sequence Gly-X-Y in its NH_2-terminal portion [6]. The gross structure of S-MBP is remarkably homologous to that of C1q, a complement component [7]. S-MBP has been identified as a serum factor associated with self-defence system. Thus, S-MBP is able to activate complement through the classical pathway [8–10] and also functions as an opsonin by itself [11]. In addition, a common deficiency in complement derived opsonin, occurring in 5–7% of the population, is associated with low serum levels of MBP [12, 13]. In our previous studies, S-MBP was shown to lyse sheep red blood cells, to which yeast mannan had been bound, in the presence of complement [8, 9]. Furthermore, S-MBP has complement-dependent bactericidal activity [14]. Thus, *Escherichia coli* K12 and B strains, which have exposed *N*-acetylglucosamine and L-glycero-D-manno-heptose, respectively, are killed by S-MBP with the help of complement.

In this study, we tried to determine if S-MBP is cytotoxic to mammalian cells in a complement dependent manner. To this end, we used baby hamster kidney (BHK) cells which had been treated with an α-mannosidase inhibitor, 1-deoxymannojirimycin (dMM). dMM appears to inhibit α-mannosidase I, thereby blocking the conversion of high

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mannose-type oligosaccharides to complex-type oligosaccharides [15].

Materials and methods

Materials

Rabbit serum MBP was isolated from pooled sera with an affinity column of Sepharose 4B-mannan as described previously [1]. Guinea pig complement, preimmune guinea pig serum and FITC-conjugated goat anti-guinea pig IgG were purchased from Organon Teknika Corp. (West Chester, PA). $Na_2{}^{51}CrO_4$ was purchased from ICN Radiochemicals (Irvine, CA). 1-Deoxymannojirimycin (dMM) was kindly provided by Nippon Shinyaku Co. Ltd (Kyoto, Japan). Agarose was purchased from Dojindo Laboratories (Kumamoto, Japan) and poly-L-lysin from Sigma Chemical Co. (St Louis, MO). Guinea pig anti-rabbit MBP serum was prepared as described previously [1].

Complement

Endogenous MBP and cytotoxic factors present in guinea pig complement were removed by passage through a Sepharose 4B-mannan column at 4 °C, followed by adsorption with agarose (80 mg per ml serum) at 0 °C for 30 min. Total haemolytic complement was measured by the procedure described by Mayer [16] using sheep erythrocytes sensitized with Hemolysin. The 50% heamolytic unit, CH_{50}, is defined as the quantity of complement required for 50% lysis of 5×10^8 cells. A part of the treated complement was inactivated by heating at 56 °C for 30 min and used as a control.

Immunostaining of BHK cells

BHK cells were grown in monolayer culture at 37 °C in Dulbecco modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd, Tokyo) supplemented with 10% fetal-calf serum (FCS) and kanamycin. The cells were trypsinized and reseeded on cover glasses (18 × 18 mm) coated with poly-L-lysine. After 1 or 2 days, these cells were cultured in the presence or absence of 2 mM dMM for an additional 24 h. Normal or dMM treated cells on the cover glasses were washed with 20 mM HEPES buffered saline, pH 7.0, containing 2 mM EDTA to remove prebound MBP which can be present in FCS, and then washed with 10 mM Tris buffered saline, pH 7.8, containing 10 mM $CaCl_2$ (TBS-Ca) and 1% BSA. The cells were incubated with 5.9 μg per 50 μl of S-MBP in the presence of Ca^{2+} for 15 min at room temperature and then washed three times with TBS-Ca. S-MBP treated cells were fixed with 4% paraformaldehyde in phosphate buffered saline, pH 7.2, (PBS) for 15 min at room temperature and then washed two or three times with PBS containing 1% BSA. Fixed cells were incubated with a guinea pig anti-S-MBP IgG or preimmune guinea pig serum for 2 h at room temperature and then with FITC-conjugated anti-guinea pig IgG antiserum as the second antibody for 1 h at room temperature. To estimate nonspecific binding of S-MBP, some of the cells were washed with TBS-Ca containing 100 mM mannose before fixation.

Cytotoxicity assay

For the cytotoxicity assay, cells were seeded sparsely and grown for 24 h in DMEM as described above in the presence or absence of 2 mM dMM. After the 24 h incubation, dMM-treated and untreated cells were harvested with Hank's solution containing 0.1% EDTA and resuspended in DMEM containing 20 mM HEPES, pH 7.0, (HEPES-DMEM) supplemented with 10% FCS. Harvested cells were radiolabelled at 37 °C for 1 h with 80 μCi of $Na_2{}^{51}CrO_4$ per 10^6 cells [17], and then washed twice with 20 mM HEPES buffered saline, pH 7.0, containing 2 mM EDTA to remove the prebound MBP potentially derived from FCS. ^{51}Cr-labelled cells were resuspended in HEPES-DMEM and the number of the cells was counted. The labelled cells were suspended in reaction tubes at 1×10^4 cells per tube and mixed with S-MBP for 15 min at 4 °C in HEPES-DMEM. To the suspension, guinea pig complement was added and the mixture was incubated for 1 h at 37 °C. After incubation, tubes were centrifuged at 5000 rpm for 5 min and the ^{51}Cr released into the supernatant was measured with a Beckman Auto-Gamma spectrophotometer, Gamma-5500. Radioactivity released from the cells, which had been suspended in H_2O and repeatedly frozen and thawed, was taken as 100% release.

Results

S-MBP binds to dMM treated BHK cells

To study the effect of S-MBP on mammalian cells, a BHK cell system described by Hughes *et al.* was chosen [18]. When BHK cells were cultured in the presence of 1-deoxymannojirimycin (dMM), the concentration of complex-type oligosaccharides decreased to only 20–30% of that measured in control cells and high mannose-type oligosaccharides increased correspondingly [13]. S-MBP recognizes and binds to high mannose-type oligosaccharides but not to complex-type oligosaccharides [1, 2, 19]. It should be noted, however, that even though marked enrichment of high mannose-type oligosaccharides occurs in the dMM-treated cells, no direct evidence has been presented showing the expression of these high mannose-type oligosaccharides on cell surfaces. Thus, we tested for S-MBP binding to the surface of dMM-treated BHK cells by using an immunostaining method.

As shown in Fig. 1A, S-MBP sensitized cells which had been treated with dMM were heavily stained with anti-S-MBP IgG, whereas, S-MBP-sensitized dMM-nontreated BHK cells did not show any significant staining (Fig. 1D). Since this staining on S-MBP-sensitized dMM-treated cells was not observed when anti-S-MBP IgG was replaced with

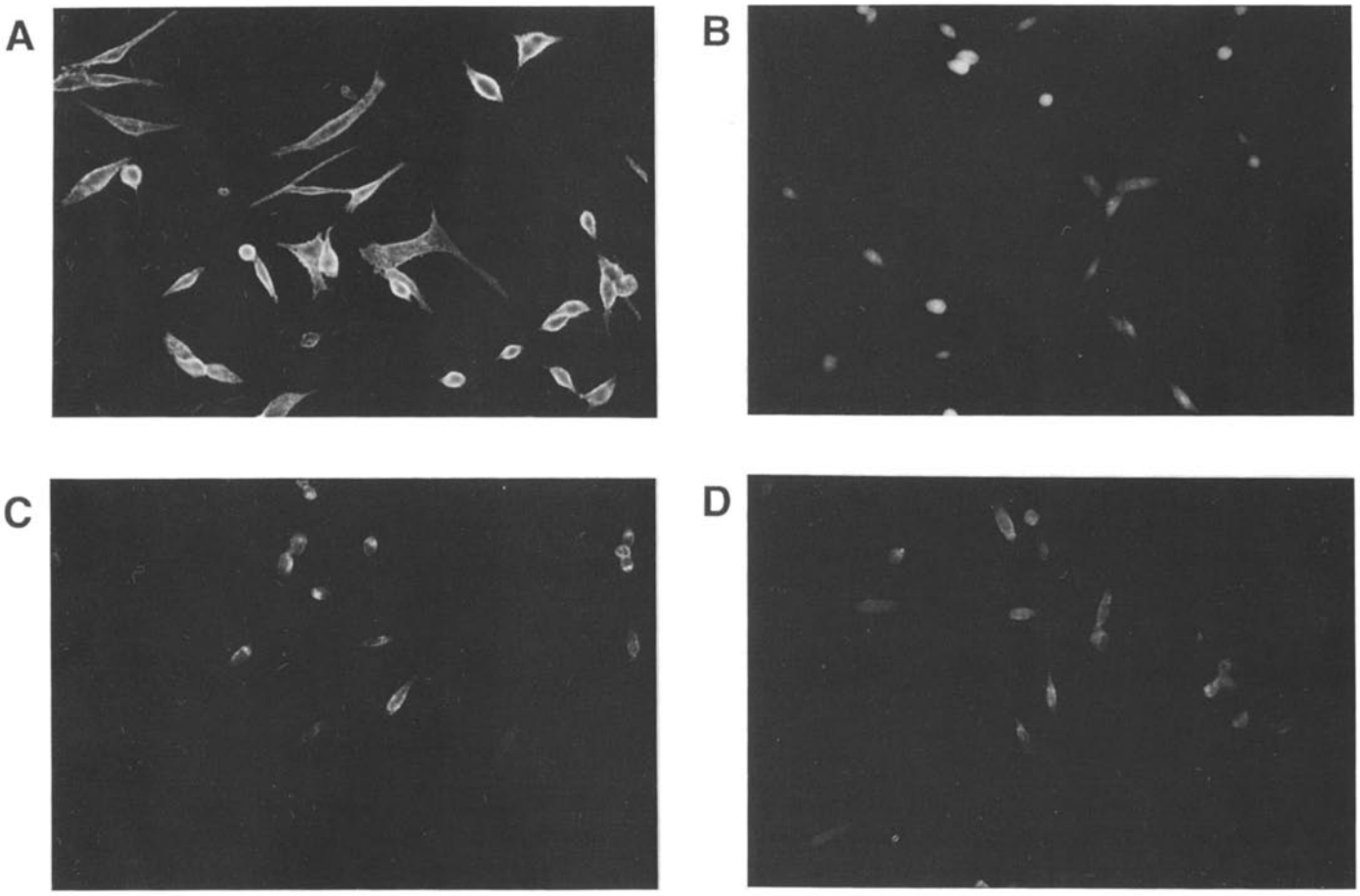

Figure 1. Immunostaining of S-MBP sensitized cells with anti-S-MBP antibody. (A) dMM treated BHK cells were sensitized with S-MBP and fixed as described in 'Materials and methods'. Fixed cells were incubated with anti-S-MBP IgG and then with FITC-conjugated anti-guinea pig IgG anti-serum. (B) The cells were incubated with preimmune serum in place of anti-S-MBP IgG. Otherwise the conditions were the same as in (A). (C) S-MBP-sensitized dMM-treated cells were washed with TBS-Ca buffer containing 100 mM mannose before fixation. (D) Normal BHK cells were treated as in (A).

preimmune serum (Fig. 1B), and disappeared when cells were washed with buffer containing 100 mM mannose after sensitization (Fig. 1C), the binding of S-MBP to the cells was specific and carbohydrate dependent.

From these observations, we concluded that dMM-treated BHK cells express high mannose-type oligosaccharides on their surfaces that specifically recognize and bind S-MBP, whereas dMM-untreated cells have no MBP-reactive carbohydrate chains on their surfaces.

Cytotoxic activity of S-MBP towards dMM-treated BHK cells in the presence of complement

In this experiment, we studied complement-mediated cytotoxicity of S-MBP by measuring the release of ^{51}Cr. As shown in Fig. 2, specific release of ^{51}Cr from S-MBP sensitized cells was observed in the presence of complement and this release increased in a dose dependent manner. Since ^{51}Cr release was maximal at 7.5 CH_{50} units of complement, we used this concentration of complement in the following assays. Heat-inactivated complement, even at high doses, had only weak cytotoxic activity, which may be due to heat-insensitive cytotoxic reagents remaining in the agarose-treated complement.

The complement induced ^{51}Cr release from S-MBP sensitized cells reached a plateau at 80% of the maximal release. All the ^{51}Cr was not released because complement-dependent cell lysis is due to increased permeability of plasma membranes and only cytoplasmic and not intracellularly bound ^{51}Cr is released. On the other hand, 100% release of radioactivity was obtained by repeated freezing and thawing under hypotonic conditions, which not only permeabilizes the plasma membranes but also destroys intracellular membrane structures. Under these conditions ^{51}Cr is released from the cytosol and intracellular organelles.

As shown in Fig. 3, sensitization of dMM-treated cells with increasing amounts of S-MBP resulted in increased release of radioactivity. Maximal release was obtained with a concentration of S-MBP as low as 0.2 $\mu g\,ml^{-1}$. When normal complement was replaced with heat-inactivated complement, S-MBP-mediated cytotoxic activity decreased

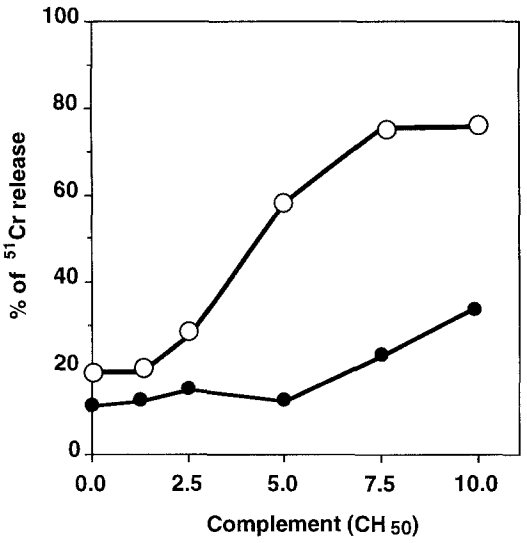

Figure 2. Complement dependent ^{51}Cr release from S-MBP sensitized cells. dMM treated BHK cells labelled with ^{51}Cr (1×10^4 cells per tube) were sensitized with S-MBP for 15 min at 4 °C in HEPES-DMEM. To the sensitized cells, an increasing amount of guinea pig complement (○) or heat inactivated complement (●) was added. The cells were then incubated for 1 h at 37 °C. The reaction mixtures were centrifuged at 5000 rpm for 5 min and the radioactivity in the supernatant was measured.

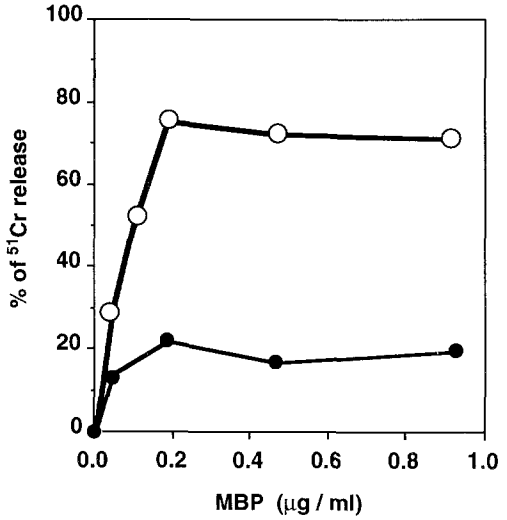

Figure 3. S-MBP dependent ^{51}Cr release in the presence of complement. dMM treated BHK cells labelled with ^{51}Cr (1×10^4 cells per tube) were sensitized with various amounts of S-MBP for 15 min at 4 °C in HEPES-DMEM. To the cells, 7.5 CH_{50} units of guinea pig complement (○) or heat inactivated complement (●) was added and the suspension was incubated for 1 h at 37 °C. The reaction mixtures were centrifuged and the radioactivity in the supernatant was measured.

to the control level. These results indicate that the cytotoxic activity of S-MBP is mediated by complement. In addition, sensitization in the absence of MBP resulted in no specific release of radioactivity even in the presence of $10CH_{50}$ units of complement, ruling out the possibility that dMM-induced modification of cell surface carbohydrates *per se* is associated with the activation of complement.

Discussion

In this experiment, we demonstrated for the first time that in the presence of complement S-MBP was cytotoxic not only to microorganisms but also to mammalian cells which have mannose-rich carbohydrates on their surfaces. These results confirm and extend the role of S-MBP as a self defence factor which discriminates self and nonself on the basis of the structure of cell surface oligosaccharides. Most circulating blood cells are covered with complex-type oligosaccharides and generally do not have high mannose-type oligosaccharides that can bind circulating S-MBP. We confirmed this fact by using FACS (fluorescence activated cell sorter) analysis with FITC (fluorescein isothiocyanate) labelled S-MBP of cells collected from mouse peripheral blood (data not shown). However, the expression of high mannose-type oligosaccharides on the surfaces of cells may occur with viral infections or oligosaccharide processing disorders caused possibly by malignant transformations. For example, Ezekowitz *et al.* showed that human MBP inhibits HIV infection of T-cell lines *in vitro* [20]. They demonstrated that human MBP binds to the carbohydrate chains of recombinant viral protein, gp120 and the surface of HIV infected T-cell lines. In addition, some colorectal carcinoma cell lines, such as LS180, SW1116 and WiDr, are stained with anti-S-MBP IgG in a similar manner as dMM-treated BHK cells (unpublished observation).

S-MBP functions as a direct opsonin [11], and Malhotra *et al.* reported enhanced phagocytosis when human leukocyte C1q receptor binds the collagen-like domains of S-MBP [21]. It should be noted, however, that the concentration of MBP required to show this direct opsonin activity ($>50\ \mu g\ ml^{-1}$) is much higher than that required for complement activation ($>0.2\ \mu g\ ml^{-1}$). This latter concentration is lower than the average physiological level of MBP in human plasma (approximately $1.8\ \mu g\ ml^{-1}$) [22].

Clinical studies have demonstrated an association between low serum levels of S-MBP and immune opsonic deficiency [12, 13]. This opsonic defect, which impairs phagocytosis of complement sensitized zymosan by polymorphonuclear leukocytes, occurs in 5–7% of the population and is characterized by vulnerability to a range of microorganisms. The disorder is familial. More recently, it was shown that low serum levels of MBP correlated with the occurrence of a single point mutation of the S-MBP gene in its collagen-like domain, which resulted in replacement of Gly54 by Asp54 [23, 24] and Gly57 by Glu57 [25]. These replacements appear to inhibit the formation of the normal triple helix structure in the collagen-like domain of the molecule

[26, 27]. S-MBP with these mutations still possesses binding activity to its sugar ligands but has lost the ability to initiate the classical complement pathway.

With regards to the mechanisms of activation of complement by S-MBP, we and others [9, 10] have shown that the activation involves the binding of $C1r_2s_2$ to S-MBP and subsequent conversion of C1s from a proenzyme to an active protease by limited proteolysis. This carbohydrate-mediated complement activation takes place via the well established classical pathway except that S-MBP is responsible for the initiation of the activation reaction, in which S-MBP plays the role of C1q in the immune complex-mediated complement activation. More recently, a new complement component which can also initiate S-MBP mediated complement activation, MBP-associated serine protease (MASP), was isolated [28].

From these observations, it is reasonable to conclude that S-MBP can eliminate unusual host cells such as viral infected cells or transformed cells whose surfaces are covered by high mannose-type oligosaccharides, by activating complement through the classical pathway.

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