Communication

Serum Lectin with Known Structure Activates Complement through the Classical Pathway*

(Received for publication, November 20, 1986)

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Serum mannan-binding protein (MBP), a lectin specific for mannose and N-acetylglucosamine, was revealed to activate the complement system as measured by passive hemolysis using sheep erythrocytes coated with yeast mannan. In contrast, rat liver MBP, which shares many properties in common with serum MBP, could not activate complement at all. The activation by serum MBP was inhibited effectively by the presence of haptenic sugars and dependent absolutely upon the presence of C4, indicating that the activation is initiated by the sugar binding activity of MBP and proceeds through the classical pathway. The 25 NH₂-terminal amino acid sequence of rat serum MBP determined in this study was completely matched with that of MBP A deduced from cDNA sequence by Drickamer et al. (J. Biol. Chem. 261, 6878–6887), revealing that MBP A is in fact identical with serum MBP. On the basis of the knowledge of primary structures and physicochemical properties of rat serum and liver MBPs, a possible mechanism of the complement activation by serum MBP is discussed with reference to close similarity in the gross structures of serum MBP and C1q.

In our previous studies, a protein (lectin) capable of recognizing mannose and N-acetylglucosamine was isolated from various mammalian sera and was called serum mannan-binding protein (MBP) (1–4). Serum MBP is a macromolecule with an apparent molecular mass of ~700,000 daltons consisting of multimers of a single subunit of around 30,000 daltons and has a collagen-like structure in the molecule (4, 5). Despite the widespread occurrence in mammals, physiological significance of this lectin is not yet known. On the other hand, C-reactive protein, a nonimmunoglobulin protein with lectin-like activity, has been shown to activate complement (6, 7). In addition, the presence of bactericidal factors in nonimmune animal sera has also been documented. These factors have sugar binding activities and kill bacterium by the aid of complement (8, 9). These observations prompted us to investigate the capability of the serum MBP to activate the complement system.

We report herein that purified serum MBP from various sources activates complement via the classical pathway and discuss possible mechanisms of the complement activation with regards to the unusual structure of serum MBP.

EXPERIMENTAL PROCEDURES

Materials—Saccharomyces cerevisiae mannan was prepared as described previously (10) from bakers' yeast purchased from Oriental Yeast Company, Tokyo. Sepharose 4B-mannan was prepared as described previously (11). MBP was isolated from human and rabbit sera, and rat liver by using an affinity column of Sepharose 4B-mannan as described previously (1, 3, 11). Two MBPs were isolated and characterized from rat serum with the results published elsewhere (2). The major component had an apparent molecular mass of ~650,000 daltons (MBP-II), the size corresponding to those of human and rabbit serum MBPs, whereas the minor component of approximately 200,000 daltons (MBP-I) was indistinguishable from the liver MBP in many properties. Unless otherwise stated, rat serum MBP (MBP-II) was used in the following experiments.

Complement—Guinea pig serum was employed as a complement source. Fresh freeze-dried guinea pig serum was obtained from the Society for the Study of Microbiology of Osaka University. C4-deficient guinea pig serum was prepared from C4-deficient guinea pigs (12). Before use all sera were absorbed by passage at 4 °C through Sepharose 4B-mannan columns to remove endogenous MBP. Three milliliters of serum was absorbed per ml column bed volume; under these conditions 20–30% of the complement activity was lost. Total hemolytic complement was measured by the procedure described by Mayer (13) using sheep erythrocytes treated with hemolysin. Guinea pig C4 was purchased from Damedico Co., Miami, FL.

Passive Hemolysis—Sheep erythrocytes were washed in saline and brought to 5% packed cell volumes. These were coated with mannan by the chromatium chloride method (14) essentially as described by Osman et al. (6); 0.5-ml aliquots of mannan solution (5–150 μg) were mixed with an equal volume of CrCl₃ solution (0.5 mg/ml), an equal volume of the erythrocyte suspension (1 × 10⁸ cells) was added, and the mixture was incubated with occasional mixing for 5 min at 25 °C. The reaction was stopped by adding 1.5 ml of ice-cold GVB (gelatin-Veronal-buffered saline, 5 mM Veronal buffer, pH 7.4, containing 0.145 M NaCl, 0.1% gelatin, 2 mM CaCl₂, and 0.5 mM MgCl₂). The erythrocytes coated with mannan (ME) were washed three times with centrifugation with GVB, resuspended to a final concentration of 1 × 10⁹ cells/ml in GVB. To test for passive hemolysis of ME, 0.1 ml of cell suspensions (1 × 10⁶ cells) was added to 0.4 ml of MBP (1–1000 ng) in GVB and incubated with gentle shaking for 15 min at room temperature; the cells were washed in ice-cold GVB and resuspended to 1 × 10⁹ cells/ml. The cells sensitized with MBP (1 × 10⁶ cells) were incubated with MBP-depleted complement diluted with GVB in a final volume of 1.5 ml. The mixtures were incubated at 37 °C for 1 h and centrifuged, and A₅₄₀ of the supernatant was determined. The degree of specific lysis was calculated based on the absorption of an equivalent volume of cells totally lysed in water and is expressed as a percentage. Correction was made for the value observed in the absence of MBP, which was always lower than 5% of the totally lysed cells' value.

Inhibition of MBP-dependent Hemolysis by Various Sugars and Mannan—Effects of sugars and mannan on MBP-dependent hemolysis of ME were assayed in two ways. In one experiment, increasing amounts of various sugars (~200 mM) or mannan (~100 μg) were added to the ME suspension (1 × 10⁹ cells coated with 1.0 μg of mannan) immediately before the addition of MBP (1.0 μg), and the mixture (0.5 ml) was incubated at 37 °C for 15 min at room temperature. The cells were then washed and assayed for hemolysis as described above. In the other experiment, various sugars or mannan were added to the...
ME-MBP suspension (1 × 10⁶ cells coated with 1.0 μg of mannan and sensitized with 1.0 μg of MBP), and the mixture (0.5 ml) was incubated for 2-60 min at room temperature. After washing with GVB, the cells were resuspended in GVB, mixed with MBP-depleted complement, and assayed for hemolysis as described above.

**Determination of Amino Acid Sequence—Reduction and carboxymethylation of MBP-II isolated from rat serum were carried out as described by Hirai (15). The reduced and carboxymethylated MBP-II was applied to a sequencer (Model 470A of Applied Biosystems), and the amino acid derivatives released were determined by a PTH analyzer (Model 120A of Applied Biosystems).**

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**RESULTS**

**Complement-dependent Hemolysis of ME Sensitized with Serum MBP**—Sensitization of washed ME with serum MBP resulted in cells (ME-MBP) that were readily lysed upon incubation at 37 °C for 1 h with guinea pig serum from which endogenous MBP had been depleted. The dose-response curve for lysis of such cells by guinea pig complement after sensitization with MBP is shown in Fig. 1A. Increased lysis was observed as the amount of the complement increased. The presence of more than 1 CH₅₀ led to a 100% lysis if the cells were sensitized with sufficient amounts of MBP. For most of the subsequent experiments, 2 CH₅₀ units were used.

The dependence of this reaction on both mannan and MBP levels was further characterized. It can be seen in Fig. 1B that lysis was greater as larger amounts of mannan were used for coating: 1-3 μg of mannan/10⁶ cells was optimal for lysis of erythrocytes sensitized with 5-500 ng of serum MBP in the presence of 2 CH₅₀. It can also be seen in Fig. 1C that increased lysis was observed as the amounts of MBP used for sensitization were increased, and 0.1-1 μg of MBP/10⁶ cells was used for most of the subsequent experiments.

**Hemolysis by MBP Depends on the Presence of C4**—Guinea pig serum (complement) genetically deficient in C4 was tested for its ability to support lysis. As shown in Fig. 2A, C4-deficient serum was unable to lyse ME sensitized with MBP, and this activity could be completely reconstituted by the addition of purified C4 as shown in Fig. 2B. These results confirmed that this lysis is indeed complement-dependent and revealed that the activation of complement proceeds through the classical pathway rather than the alternative pathway, the latter being known to become operable without involvement of C4 (16).

**Inhibition of MBP-dependent Hemolysis of ME by Haptenic Sugars and Mannan**—To determine whether the lysis observed was indeed mediated by the sugar binding activity of MBP or not, various sugars were co-incubated with MBP at the step of sensitization and their ability to inhibit lysis was estimated. When tested at the sugar concentration of 5 mM, where mannose gave rise to 50% inhibition, those sugars known to be potent inhibitors of MBP binding, L-fucose, N-acetylmannosamine, and N-acetylgalcosamine inhibited the lysis close to or more than 50%. In contrast, galactose, a noninhibitor of MBP binding, did not inhibit the lysis at all. It should be noted, however, that if these inhibitory sugars were added after completion of the sensitization with MBP, they inhibited the lysis partially; only less than 40% inhibition was observed in the presence of 100 mM mannose. The same results were obtained for mannann. An excess amount of mannann (>10 μg per tube) when added at the step of sensitization with MBP inhibited the lysis completely, whereas when added after sensitization, inhibition was only partial (~45%) even in the presence of 100 μg of mannann. This incomplete inhibition is not ascribed to the slow rate of dissociation of ME-MBP complexes, since alteration of the incubation time from 2 to 60 min did not change the results. This observation is in sharp contrast with the reversible binding reaction between MBP and mannan which had been immobilized on Sepharose 4B resins, the reaction being routinely utilized in the purification procedure for the MBP. The reason for this apparent discrepancy is currently unknown. It may suggest the presence of alternative binding site(s) on the MBP molecule which is probably specific to cell surfaces.

**Complement Activation by MBP from Other Sources**—As shown in Table I, MBPs isolated from rat, rabbit, and human sera lysed ME dose-dependently in the presence of guinea pig complement; the levels of the activity were virtually the same throughout the species. In contrast, a similar lectin isolated from rat liver (liver MBP) (11), was unable to lyse ME and so was conglutinin isolated from bovine serum (4, 17-19), despite the fact that they have sugar binding specificities and molecular structures very similar to those of the serum MBPs.
It should be noted that the activation of complement by MBP was observed not only in heterologous combinations as shown in Table I but also in autologous combinations; human complement (MBP-depleted human serum) could be activated by human serum MBP dose-dependently.

**Primary Structure of Serum MBP**—With regard to the differential abilities to activate complement between the liver and serum MBPs, we attempted to determine the primary structure of MBP isolated from rat serum (MBP-II) and compare it with that of the liver MBP, which had already been established by Drickamer et al. (20) and our group (21). NH$_2$-terminal amino acid sequence of serum MBP was determined by protein sequencer with the results shown in Fig. 3. To our surprise, the 25 NH$_2$-terminal amino acid sequence was completely identical to that of MBP-A described by Drickamer et al. (20). They determined the complete amino acid sequence of MBP-A from the cDNA sequence. However, the natural location and biological function of the protein remained to be elucidated. We have now clearly established that MBP-A is in fact the serum MBP.

**DISCUSSION**

As was pointed out by Drickamer et al. (20), the liver and serum MBPs (MBP-C and MBP-A, respectively, according to Drickamer et al.) shared common structural features such as the presence of a collagen-like domain consisting of 18–20 repeats of the sequence Gly-X-Y and overall sequence homology of the mature proteins as high as 56% with only four gaps. Despite these similarities, however, there are some distinctions between the two MBPs which may be related to their differential functions. The serum MBP is a macromolecule with an apparent molecular mass of 650,000 daltons, consisting of approximately 20 identical subunits of 31,000 daltons, which are linked together by disulfide bonds, whereas the liver MBP is a 200,000-dalton protein, consisting of 6 identical subunits of 32,000 daltons, and occurs as disulfide-linked dimers and trimers of the subunit under denaturing conditions (10). The other characteristic of the serum MBP is the presence of the sequence Arg-Gly-Asp-Ser (amino acid 68–71 (20), which is known to be the cell attachment site of fibronectin (22). This sequence might be associated with the sugar-noninhibitable binding of the serum MBP to ME as described above, although no evidence is available to show the interaction of erythrocytes and fibronectin at the moment.

Interestingly, the gross structure of the serum MBP is remarkably homologous with that of C1q, a constituent of complement which binds directly to immune complexes to initiate the activation of the classical pathway. C1q is a macromolecule ($M_0 = 410,000$) consisting of 18 subunits of three different polypeptides (A, B, and C chains, $M_0 = 23,000$), linked together by disulfide bonds located at their NH$_2$-terminal portions. They have a collagen-like domain with 25 repeats of the sequence Gly-X-Y, to which other C1 components (Clr and Cls) are believed to bind, leading to the activation of the “complement cascade” (23). Taking these structural resemblances into consideration, it may be conceivable that serum MBP, once fixed on cell surfaces, functions as C1q does to initiate the activation of the classical pathway. On the other hand, however, one should also consider the alternative possibility that C1q is bound to ME-MBP complexes to initiate complement activation as are the cases with antigen-antibody complexes and C-reactive protein-pneumococcal C-polysaccharide complexes (7).

A complement-dependent bactericidal factor (RaRF) isolated from mouse serum, which is specific for the Ra chemo- 

type strain of Salmonella, shares some properties with serum MBP. Thus, RaRF recognizes N-acetylglucosamine, requires calcium for binding, and consists of one major component ($M_0 = 28,000$) (8, 9). However, lack of detailed information on the structure of RaRF precludes closer comparison. It should be emphasized here that the complement activating activity described in this study is clearly distinct from that of C-reactive protein. Thus, human serum MBP used in this study is

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**TABLE I**

Complement-dependent passive hemolysis by various MBPs

| Species | MBP | MBP added |
|---------|-----|-----------|
|         |     | 1 ng | 10 ng | 100 ng |
| Rat     | Liver | 0 | 0 | 0 |
|         | Serum | 2.6 | 25.7 | 100 |
| Rabbit  | Serum | 3.6 | 29.7 | 100 |
| Human   | Serum | 13.0 | 67.5 | 100 |
| Bovine  | Conglutinin | 0 | 0 | 3.3 |

FIG. 3. NH$_2$-terminal amino acid sequence of MBP-II isolated from rat serum.

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$^a$ T. Kawasaki and I. Yamashina, unpublished observations.
Complement-activating Serum Lectin completely free from C-reactive protein, and C-reactive protein, if present, cannot bind mannan (4).

Acknowledgments—We thank Dr. K. Inoue of Osaka University for providing us with C4-deficient guinea pig serum and for helpful suggestions and A. Ohashi of Applied Biosystems Japan, Co., Osaka, for performing the amino acid sequence analysis.

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