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Binding of Mannose-Binding Protein to *Klebsiella* O3 Lipopolysaccharide Possessing the Mannose Homopolysaccharide as the O-Specific Polysaccharide and Its Relation to Complement Activation

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Lipopolysaccharide from *Klebsiella pneumoniae* O3, which possesses the mannose homopolysaccharide as the O-specific polysaccharide, exhibits an extraordinarily high ability to activate the human complement system. We isolated the mannose-binding protein with a *Klebsiella* O3 lipopolysaccharide affinity column. The protein isolated had a molecular mass of much higher than 200 kDa, and it consisted of subunits with an apparent molecular mass of 32 kDa. The NH_2-terminal sequence of the 32-kDa subunits was completely consistent with a part of the amino acid sequence of human serum mannose-binding protein. In immunoblotting, an anti-mannose-binding protein monoclonal antibody was definitely reactive with the isolated protein with the higher molecular mass. The protein isolated was bound exclusively to lipopolysaccharides possessing the mannose homopolysaccharide, not to lipopolysaccharide possessing the heteropolysaccharides. *Klebsiella* O3 lipopolysaccharide did not exhibit a high anticomplement activity in the serum from which the mannose-binding protein was depleted. It was concluded that the serum factor that bound to *Klebsiella* O3 lipopolysaccharide may be mannose-binding protein and that it may play a crucial role in the strong complement activation by *Klebsiella* O3 lipopolysaccharide.

Lipopolysaccharide (LPS) can activate the complement system in various species by the classical and alternative pathways in vivo and in vitro. It is now well established that the two pathways are activated by different, nonoverlapping portions of the LPS molecule. The lipid A region of the LPS molecule leads to an antibody-independent activation of the classical pathway, and the polysaccharide portion activates the alternative pathway by a lipid A-independent, antibody-independent mechanism (4, 17, 23). Thus, LPS can directly activate the complement system through both pathways. Recently, several serum factors that bind LPS and act as complement activation factors have been purified and partially characterized (1, 9). While those factors recognized relatively conserved LPS core oligosaccharide determinants, they seem to activate the classical pathway by different mechanisms (23).

Previously we found that LPS isolated from *Klebsiella pneumoniae* O3 exhibited an extraordinarily high anticomplement activity in a hemolysis assay using human sera (28). The activity of LPS from *Klebsiella* O3 to activate human complement was more than 100 times higher than that of LPS from *Escherichia coli* O111, *E. coli* O55, or *Salmonella enteritidis*. The free lipid A isolated from *Klebsiella* O3 LPS by acid hydrolysis and the R-form LPS from a mutant lacking the O-specific polysaccharide portion exhibited much lower anticomplement activity. It was therefore suggested that the O-specific polysaccharide moiety markedly enhanced the complement activation by *Klebsiella* O3 LPS. It is of particular interest that LPS from *K. pneumoniae* O3 possesses the same linear mannose homopolysaccharide as the O-specific polysaccharide (2). Moreover, the other LPSs possessing mannose homopolysaccharides, such as LPSs from *Klebsiella* O5, *E. coli* O8, and *E. coli* O9, also exhibited a high anticomplement activity (28). It was indicated that the mannose homopolysaccharide moiety of the LPS molecule may be crucial for expression of an extraordinarily high complement activation by those LPSs. In this study, we attempted to isolate the mannose-binding protein (MBP) which may bind the O-specific polysaccharide moiety of *Klebsiella* O3 LPS. Here, we describe the participation of MBP in the strong complement activation by *Klebsiella* O3 LPS.

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MATERIALS AND METHODS

LPS. *Klebsiella* O3 LPS was extracted from *K. pneumoniae* LEN-1 (O3:K1$^-$) by the phenol water method (24, 29) and purified by ultracentrifugation. LPS was precipitated by addition of ethanol containing 0.2% $MgCl_2$ to a final concentration of 67% and then washed with ethanol. LPSs from *Klebsiella* O5, *Klebsiella* O7, *E. coli* O8, and *E. coli* O9 were also prepared (28). LPSs from *E. coli* O55, *E. coli* O111, and *S. enteritidis* were obtained from Difco Laboratories, Detroit, Mich.

Antibody. An anti-human MBP monoclonal antibody (MAb) was generously provided by Toshisuke Kawasaki, Kyoto University. The MAb was reactive with human 690-kDa MBP but not with the 32-kDa subunit. It was used at a dilution of 1:200 in all experiments.

Preparation of *Klebsiella* O3 LPS-Sepharose 6B. *Klebsiella* O3 LPS-Sepharose 6B was prepared by using epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) (5). *Klebsiella* O3 LPS (100 mg) was suspended with 0.1 M NaOH at an LPS concentration of 5 mg/ml. After centrifugation at 3,000 × *g* for 15 min to remove large aggregates, it was applied to epoxy-activated Sepharose 6B which had been washed with 0.1 M NaOH. The mixture of *Klebsiella* O3 LPS (20 ml) and epoxy-activated Sepharose 6B (15 ml) was incubated for 16 h at 40°C. The gel suspension was washed with 0.1 M $NaCO_3$ buffer (pH 8.0) and then with 0.1 M acetic acid. On the following day, the residual active groups were blocked with buffer containing 1 M ethanolamine. The suspension was washed with 0.1 M acetic acid (pH 4.0) containing 0.5 M NaCl and then with distilled water. The conjugation of *Klebsiella* O3 LPS on epoxy-activated Sepharose 6B was confirmed by an enzyme-linked immunoabsorbent assay (ELISA) with a rabbit anti-*Klebsiella* O3 LPS antibody. Finally, it was poured into a chromatographic column (3 by 5 cm), and the column was washed with loading buffer (50 mM Tris-HCl buffer [pH 7.8], 1 M NaCl, 20 mM $CaCl_2$) until the optical density at A_{280} was less than 0.01.

Purification of MBP. The techniques described by Lu et al. (12) were used as the basis for the isolation of MBP. All steps were carried out at 4°C unless otherwise stated. Frozen pooled normal human serum (150 ml) was thawed and

dialyzed extensively against loading buffer without $CaCl_2$. The dialyzed solution was made 50 mM with respect to $CaCl_2$ and centrifuged at $15,000 \times g$ for 20 min. The supernatant was filtered through a Milex-HV 0.45-μm-pore-size filter unit (Millipore, Bedford, Mass.) and applied to a *Klebsiella* O3 LPS-Sepharose 6B column equilibrated with loading buffer. The column was washed with loading buffer until unbound proteins were removed and then eluted with 50 mM Tris-HCl (pH 7.8) containing 1 M NaCl and 10 mM EDTA. The eluate (10 ml) was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and then applied to an Econo-Pac Q cartridge (Bio-Rad Laboratories, Hercules, Calif.) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The bound proteins were eluted with a linear gradient from 0 to 600 mM NaCl (FPLC [fast protein liquid chromatography] system; Pharmacia Biotech, Uppsala, Sweden). The MBP-containing fractions which were eluted between 170 and 290 mM NaCl were combined and concentrated to 300 μl by ultrafiltration with a Centricon-30 (Amicon, Inc., Beverly, Mass.). The concentrated eluate was injected into an FPLC-Superose 6 (HR 10/30) column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, 1 mM EDTA, and 0.05% (wt/vol) Tween 20. The fractions eluted between 9 and 12 ml were concentrated to 300 μl by ultrafiltration and passed through an anti-human immunoglobulin M column (E. Y. Laboratories, Inc., San Mateo, Calif.).

Gel electrophoresis. The samples were suspended in sample buffer containing 1% sodium dodecyl sulfate (SDS) in the presence or absence of 1% 2-mercaptoethanol and boiled for 2 min. Samples were separated by 5 to 20% gradient polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS under reducing or nonreducing conditions. Protein bands were stained with Coomassie brilliant blue in 10% trichloroacetic acid. The prestained high- and low-molecular-weight standard markers from Nippon Bio-Rad Laboratories, Tokyo, Japan, were used as references.

Immunoblotting. The protein isolated was separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). The transferred protein was blocked with 5% skim milk and washed four times with 0.01 M phosphate-buffered saline, pH 7.2 (PBS), containing 0.05% Tween 20 (T-PBS), and then the membrane was treated with the anti-MBP MAb. The membrane was washed with T-PBS, and then resulting immune complexes were detected with peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Dako, Kyoto, Japan). Finally, labeled antigen bands were detected by enhanced chemiluminescence Western blotting (immunoblotting) detection reagents (Amersham, London, England).

Binding of MBP to LPS. The binding of MBP to LPS was detected by ELISA. Microtiter plates were coated with *Klebsiella* O3, O5, and O7, *E. coli* O8, O9, O55, and O111, and *S. enteritidis* LPSs (21) and then washed three times with T-PBS; unbound sites were blocked by the addition of 5% skim milk to wells for 1 h at 37°C and then washed with T-PBS. The isolated protein (0.5 or 2 μg/ml) was added and incubated in the wells for 1 h. After washing, the plates were treated with the anti-MBP MAb. Finally, the wells were treated with peroxidase-conjugated goat anti-mouse immunoglobulin G antisera (Dako) and *o*-phenylenediamine. The optical density of each well was read relative to that of a substrate blank at 492 nm on an automatic microplate reader.

Sequence of the isolated protein. The samples were electrophoresed, transferred onto polyvinylidene difluoride membranes, stained, and sequenced directly on an Applied Biosystems model 477A protein sequencer as described by Matsudaira (13).

Assay for complement activation by LPS. Various concentrations of LPS in PBS (12.5 μl) were added to human control serum or MBP-depleted serum (50 μl), and the mixture was incubated for 30 min at 37°C. MBP-depleted serum was prepared as follows. Protein A-conjugated Sepharose 4B (50 mg) (Pharmacia Fine Chemicals) was mixed with an excess of the anti-MBP MAb and washed. Human serum (1 ml) was incubated with anti-MBP MAb-conjugated protein A-Sepharose 4B for 1 h at 4°C. MBP-depleted serum was obtained by centrifugation. Control serum was treated with protein A-conjugated Sepharose 4B alone. Determination of anticomplement activity by LPS was performed with a hemolysis kit by Mayer's technique 1/2.5 (Denka Seiken Co., Tokyo, Japan) as described previously (28). Anticomplement activity of LPS at various concentrations was expressed as inhibition of hemolysis.

RESULTS

Isolation of MBP from human sera. In a typical experiment, approximately 150 ml of pooled human sera was dialyzed and applied to *Klebsiella* O3 LPS-Sepharose 6B. MBP was isolated as described in Materials and Methods. The protein isolated was analyzed by SDS-PAGE under nonreducing or reducing conditions (Fig. 1). SDS-PAGE demonstrated a broad band with a molecular mass of much higher than 200 kDa under nonreducing conditions, and it was dissociated into the subunits with an apparent molecular mass of 32 kDa under reducing conditions, consistent with the size of the monomer of MBP.

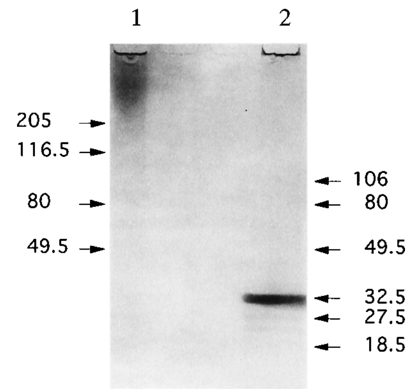

FIG. 1. Characterization of the isolated protein by SDS-PAGE under nonreducing and reducing conditions. The isolated protein was electrophoresed on a 5 to 20% gradient polyacrylamide gel. It migrates under the well as high-molecular-mass multimers under nonreducing conditions (lane 1) and migrates as monomers of 32 kDa under reducing conditions (lane 2). The positions of size markers are indicated in kilodaltons.

The NH_2-terminal sequence of the isolated protein. The isolated protein was subjected to SDS-PAGE under reducing conditions and transferred to a membrane. The 32-kDa subunits on the blots were applied to the protein sequencer. The NH_2-terminal sequence of the 32-kDa subunit was Glu-Thr-Val-Thr-Cys-Glu-Asp-Ala-Gln-Lys, consistent with a part of the amino acid sequence of human serum MBP described by Tayler et al. (22).

Characterization of the isolated protein by immunoblotting with the anti-MBP MAb. The isolated proteins were separated by SDS-PAGE under nonreducing conditions and transferred to a membrane. Their reactivity with an anti-MBP MAb which recognizes human 690-kDa MBP was studied (Fig. 2). The anti-MBP MAb recognized characteristic ladder bands with higher molecular masses in the immunoblot. The position of the bands corresponded to that of the isolated protein in SDS-PAGE under nonreducing conditions.

Binding of MBP to various kinds of LPS. Binding of the isolated protein to various kinds of LPS was determined by ELISA using the anti-MBP MAb (Table 1). The protein isolated was added to wells precoated with various kinds of LPS.

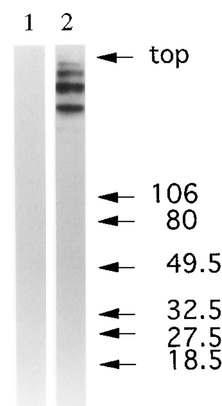

FIG. 2. Immunoblotting of the isolated protein with the anti-MBP MAb. The membranes were treated with irrelevant control sera (lane 1) or the anti-MBP MAb (lane 2). Note that the anti-MBP MAb recognized characteristic ladder bands with high molecular masses. The positions of size markers are indicated in kilodaltons.

TABLE 1. Binding of MBP to various kinds of LPS[a]

LPS	Optical density at 492 nm
Klebsiella O3	0.388
Klebsiella O5	0.294
E. coli O8	0.322
E. coli O9	0.386
Klebsiella O7	0.019
E. coli O55	0.002
E. coli O111	0.013
S. enteritidis	0.022
Negative control	0.007

[a] Microplates were precoated with various kinds of LPS and incubated with MBP (2 μg/ml). MBP bound was detected by ELISA using the anti-MBP MAb. The negative control was precoated with *Klebsiella* O3 LPS and incubated with no MBP.

The wells coated with LPS from *Klebsiella* O3, *Klebsiella* O5, *E. coli* O9, or *E. coli* O8 showed positive reactions in ELISA, indicating that the protein isolated was bound to LPS possessing mannose homopolysaccharides. However, there was no positive reaction in the wells coated with LPS from *Klebsiella* O7, *E. coli* O55, *E. coli* O111, or *S. enteritidis.*

Participation of MBP in complement activation by *Klebsiella* O3 LPS. To study the participation of MBP in complement activation by *Klebsiella* O3 LPS, the levels of complement activation of *Klebsiella* O3 LPS in control and MBP-depleted sera were compared (Table 2). *Klebsiella* O3 LPS did not exhibit a strong anticomplement activity in MBP-depleted serum, although it definitely activated the complement system in control serum. The ability of *Klebsiella* O3 LPS to activate the complement system in MBP-depleted serum was 100-fold less than that in control serum.

DISCUSSION

In this study, we isolated from human sera MBP which binds *Klebsiella* O3 LPS. Several lines of evidence suggested that the protein isolated was MBP. First, its molecular mass was higher than 200 kDa in SDS-PAGE under nonreducing conditions, and it was dissociated into the subunits with an apparent molecular mass of 32 kDa in SDS-PAGE under reducing conditions; second, it definitely reacted with the anti-MBP MAb in immunoblotting; third, its partial NH_2-terminal amino acid sequence was completely consistent with that of human serum MBP; finally, it was bound specifically to LPS possessing the mannose homopolysaccharide.

Serum MBP is a C-type lectin which binds in the presence of calcium ions to mannan and is able to trigger complement activation through binding to pathogens possessing mannose (3, 6, 10, 18, 20). Recently, it has been reported that serum MBP acts in association with a C1s-like serine protease which consumes C4 and C2, resulting in complement activation via the lectin pathway (7, 14). These findings suggest that *Klebsiella* O3 LPS possessing mannose homopolysaccharide activates the lectin pathway by using MBP. Morrison and Kline demonstrated that *E. coli* O111 LPS activated the alternative pathway and that activation was dependent on the presence of the polysaccharide moiety of LPS (16). They also suggested that the length and composition of the polysaccharide moiety of LPS could influence the ability of LPS to activate the alternative pathway. Thus, it has been accepted that the polysaccharide moiety of LPS can activate the alternative pathway (17, 23). In contrast, the present study has demonstrated that LPS possessing the polysaccharide structure may activate the complement system through the lectin pathway by using MBP. Previously we found that LPS possessing mannose homopolysaccharides as O-specific polysaccharides exhibited an extraordinarily high anticomplement activity, and its activity was more than 100 times greater than that of LPS possessing the heteropolysaccharide (28). This extraordinarily high anticomplement activity can be easily explained by the binding of MBP to the mannose homopolysaccharide moiety of LPS. In fact, *Klebsiella* O3 LPS did not exhibit a strong anticomplement activity in MBP-depleted serum. We also reported that LPSs possessing mannose homopolysaccharides as O-specific polysaccharides, such as LPSs from *Klebsiella* O3 and O5 and *E. coli* O8 and O9, exhibited a strong adjuvant action on the immune response to nonimmunogenic autoantigens in mice (26) and triggered the production of autoimmune lesions (25–27). The strong adjuvant action of LPSs possessing mannose homopolysaccharides was closely associated with their strong complement-activating ability (28). MBP might also play a crucial role in expression of a strong adjuvant action of LPS possessing mannose homopolysaccharides.

Kuhlman et al. (11) and Schweinle et al. (19) showed that MBP enhanced complement deposition via the alternative complement pathway on *Salmonella montevideo* that expresses a mannose-rich LPS and resulted in serum killing of these organisms, which are resistant to complement lysis in the absence of MBP. They emphasized that MBP and the complement system cooperate in the first line of defense of the nonimmune host. MBP may also be important for the clearance of released LPS from the circulation. However, Joiner et al. (8) demonstrated that long-chain polysaccharides resulted in complement activation at sites removed from the outer membrane surface. The long mannose homopolysaccharide portion of LPS might be useful for conferring serum resistance on some gram-negative bacilli.

Several serum factors which activate complement subsequent to binding core oligosaccharides of LPS have been identified. Ihara et al. (6) have identified a Ra LPS-binding serum protein with an approximate molecular mass of 300 kDa, which activates the classical pathway on binding Ra LPS in mouse serum. Recently, it became clear that a component of human Ra-reactive factor is identical to MBP (15). A 28-kDa serum factor which can bind specifically to the heptose–3-keto-D-manno-octulosonic acid inner core region of LPS and activate the classical pathway has been identified (1). Its relationship to MBP is still unclear.

TABLE 2. Activation of complement in MBP-depleted serum by *Klebsiella* O3 LPS

Serum	Inhibition of hemolysis (%)			
	5,000[a]	500	50	5
MBP depleted	94	52	26	22
Control	95	93	93	90

[a] Concentration (micrograms per milliliter) of LPS in serum.

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