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### Research Article

The human mannose-binding protein (MBP) is a multimeric serum protein that is divided into three domains, a cysteine-rich NH<sub>2</sub>-terminal domain that stabilizes the collagen alpha helix of the second domain and a third COOH-terminal carbohydrate recognition domain. Previous studies have shown that both native and recombinant human MBP bind to wild-type virulent *Salmonella montevideo* that expresses a mannose-rich lipopolysaccharide. Interaction with MBP results in opsonization and killing by phagocytes. In this report we show that low concentration of MBP (less than 10 micrograms/ml) markedly enhance complement deposition via the alternative complement pathway on *S. montevideo*. Despite structural similarities between MBP and the C1q subcomponent of the first complement component, MBP did not restore classical pathway activity to C1q-deficient serum, nor did it activate C1s when added to a mixture of C1r and C1s. In the presence of MBP the C3 bound to *S. montevideo* during incubation in serum was in the form of C3b and iC3b at a ratio of 1:2. Presensitization of *S. montevideo* with MBP rendered this normally serum resistant organism susceptible to complement-mediated killing. These results emphasize that MBP and complement cooperate in first line defense of the nonimmune host.

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# Human Mannose-binding Protein Activates the Alternative Complement Pathway and Enhances Serum Bactericidal Activity on a Mannose-rich Isolate of *Salmonella*

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## Abstract

The human mannose-binding protein (MBP) is a multimeric serum protein that is divided into three domains, a cysteine-rich NH<sub>2</sub>-terminal domain that stabilizes the collagen alpha helix of the second domain and a third COOH-terminal carbohydrate recognition domain. Previous studies have shown that both native and recombinant human MBP bind to wild-type virulent *Salmonella montevideo* that expresses a mannose-rich lipopolysaccharide. Interaction with MBP results in opsonization and killing by phagocytes. In this report we show that low concentrations of MBP (< 10 µg/ml) markedly enhance complement deposition via the alternative complement pathway on *S. montevideo*. Despite structural similarities between MBP and the C1q subcomponent of the first complement component, MBP did not restore classical pathway activity to C1q-deficient serum, nor did it activate C1s when added to a mixture of C1r and C1s. In the presence of MBP the C3 bound to *S. montevideo* during incubation in serum was in the form of C3b and iC3b at a ratio of 1:2. Presensitization of *S. montevideo* with MBP rendered this normally serum resistant organism susceptible to complement-mediated killing. These results emphasize that MBP and complement cooperate in first line defense of the nonimmune host.

## Introduction

Pathogenic microorganisms have evolved mechanisms to evade complement attack (reviewed in reference 1). In gram negative enteric bacteria, such as *Salmonellae*, the presence of a confluent lipopolysaccharide (LPS)<sup>1</sup> layer within the outer membrane is associated with complement resistance. In addition, wild type (smooth) LPS masks cognate epitopes on the outer membrane for cell surface phagocyte adhesion molecules, outer membrane-specific bacteriophages (2), and core-specific antibodies (3). Wild type LPS is composed of one

molecule of lipid A, one copy of a core oligosaccharide, and from none to more than 60 copies of the repeating *O*-polysaccharide (*O*-PS). Strains of *Salmonella* which lack the *O*-PS (rough phenotype) are generally nonpathogenic. These organisms are readily cleared from the circulation of experimental animals, are avidly ingested by phagocytes, and are directly killed by complement (4, 5). Despite the adaptations of *Salmonella* strains to avoid conventional host defenses, like antibody and complement, these organisms rarely cause life-threatening infections. This observation suggests that these organisms are effectively cleared by the host. We have recently shown that human mannose binding protein (MBP) can directly bind to wild type virulent *Salmonella montevideo* that express a mannose-rich *O*-PS. Interaction of native and recombinant MBP with this organism results in attachment, ingestion, and killing of MBP-opsonized bacteria by phagocytes in the absence of serum (6).

MBPs were first isolated from the serum of rabbits (7) and subsequently found in the serum and liver of humans (8–10) and rodents (11, 12). Analysis of human MBP, like its two rat homologues (13), reveals that the molecule consists of a globular carbohydrate recognition domain, and a collagen-like region which is stabilized by a cysteine-rich NH<sub>2</sub>-terminal domain (8). Initial experiments showed that human MBP bound yeast mannans (reviewed in reference 14) and were suggestive that MBP may have a role in host defense. Recently, we have shown that human MBP, which is an acute phase reactant (8), can prevent in vitro infection of H9 lymphoblasts by HIV by binding to the high mannose glycans expressed on the envelope glycoprotein of HIV (15). In addition, we have demonstrated that MBP can serve as an opsonin, and these findings underscore the role of MBP in first line host defense against mannose-rich pathogens (6).

MBPs and C1q have similar overall organization, although there is no detectable homology between the collagenous or noncollagenous portions of the proteins (reviewed in references 13 and 16). It therefore appeared likely that MBP would have a similar role to C1q, namely binding to target molecules to activate C1r and C1s and initiate classical complement pathway activation. In fact, Ikeda et al. (17) reported that MBP activated complement via the classical pathway to lyse mannan-coated erythrocytes. We set out to investigate whether MBP could enhance complement activation on virulent strains of *Salmonella* that are normally resistant to direct complement lysis.

In this report, we demonstrate that MBP-mediated deposition of complement on virulent *Salmonella* strains occurs via the alternative complement pathway. Surprisingly, the MBP-mediated complement deposition results in serum killing of

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1. Abbreviations used in this paper: ADS, adsorbed sera; LPS, lipopolysaccharide; MBP, mannose-binding protein; *O*-PS, *O*-polysaccharide.

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these organisms that are resistant to complement lysis in the absence of MBP.

## Methods

**Reagents and buffers.** Hanks' balanced salt solution (HBSS) containing several different concentrations of calcium and magnesium was used for these studies: HBSS with 20 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> (HBSS 20/1); HBSS with 0.15 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> (HBSS 0.15/1); HBSS with 4 mM MgCl<sub>2</sub>, 20 mM ethylene glycoltetraacetic acid (MgEGTA). Veronal-buffered saline (VBS, pH 7.35, containing 150 mM NaCl, 3.1 mM barbituric acid, and 1.8 mM sodium barbital) was used with various concentrations of Ca and Ni: VBS with 20 mM CaCl<sub>2</sub>, 1 mM NiCl<sub>2</sub> (VBS 20/1); VBS with 5 mM CaCl<sub>2</sub> (VBS 5/0).

**Bacteria.** The following bacteria were studied: *Salmonella typhimurium* (SH5771), *Salmonella montevideo* (SH5770), *Salmonella enteritidis* (SH4340), *Salmonella typhimurium* (TV119) (all obtained from P. H. Makela, Central Public Health Laboratory, Helsinki, Finland), *Salmonella adelaide* (from the American Type Culture Collection, Rockville, MD) and *Escherichia coli* (J5) (from E. J. Ziegler, University of California, San Diego, CA). Fig. 1 depicts the lipopolysaccharide structure for SH5770. Table I describes the *O*-polysaccharide of organisms used for these studies. Strains SH5771, SH4340, and SH5770, originally derived by Valtonen (18) by phage transduction with the *rfb* locus, have been compared extensively for virulence, for capacity to activate complement and for susceptibility to ingestion by macrophages. Strains SH5771 and SH4340 each have one mannose residue per *O*-polysaccharide subunit, whereas SH5770 has four mannose residues per subunit. Strain SH5771 is the most virulent in mice (19), activates the alternative complement pathway inefficiently (20), and is phagocytosed by macrophages at a slow rate (21). SH5770 is the least virulent (19), activates the alternative complement pathway efficiently (20), and is readily phagocytosed (21), whereas SH4340 occupies an intermediate position in all respects. *Salmonella adelaide* has a complete *O*-polysaccharide that contains no mannose. TV119, an Ra mutant (Fig. 1) completely lacking *O*-polysaccharide, but having normal lipid A and a complete core polysaccharide with an exposed *N*-acetylglucosamine moiety, activates complement by the alternative pathway (22). J5, an Rc mutant (Fig. 1), also lacks an *O*-polysaccharide side chain when grown in the absence of mannose and galactose. It activates the classical pathway independently of antibody (23, 24). Both TV119 and J5 are susceptible to complement-mediated serum bactericidal activity. All other *Salmonella* strains used in these experiments are resistant to serum lysis in nonimmune human serum.

Before each experiment the organisms were grown to mid-log phase in tryptic soy broth at 37°C with aeration, were washed twice in HBSS 20/1, and resuspended at OD 600 = 1.00 (1 × 10<sup>9</sup> organisms/ml) in HBSS 20/1.

**Sera.** Aliquots of normal human serum (NHS) collected from healthy, normal donors or from patients congenitally lacking complement components C7, C8, or C2 were stored at -70°C. Factor D-deficient serum prepared by the method of Lesavre and colleagues (25) was a gift from Dr. J. Zurlo, National Institutes of Health, Bethesda, MD. Serum deficient in both C1q and factor D was prepared by the method of Praz et al. (26) using preparative chromatography on a Biorex column. Absence of C1q was ascertained by measuring lysis of antibody-sensitized sheep erythrocytes in C1q- and factor D-deficient serum with and without reconstitution of C1q. Factor D deficiency was documented by lysis of rabbit erythrocytes in the deficient serum chelated with Mg-EGTA (see below) with and without added factor D. In each case the serum retained < 2% residual hemolytic activity in the absence of the missing component. In some experiments, serum was heated to 56°C for 30 min to block complement activation or was mixed with an equal volume of HBSS containing 4 mM MgCl<sub>2</sub> and 20 mM EGTA (final concentration 2 mM MgCl<sub>2</sub>, 10 mM EGTA), and heated for 30 min at 30°C to selectively block classical complement pathway activation. To remove naturally occurring antibodies and perhaps MBP from serum, *Salmonella* strains SH5770, SH5771, SH4340, and *S. adelaide* were grown to mid-log phase, then fixed with 0.25% glutaraldehyde. An aliquot of each strain was chilled on ice then used at a concentration of 5 × 10<sup>9</sup> bacteria/ml to adsorb a separate aliquot of ice-cold NHS. Adsorbed sera (ADS) stored at -70°C lost less than 20% of the starting hemolytic titer of C3.

Table I. Strain and *O*-polysaccharide Structure of Tested Organisms

Strain	<i>rfb</i> Genes originally from	<i>O</i> -antigen of LPS	Structure of <i>O</i> -PS side chain
<i>S. adelaide</i>			[Glu-Gal-Rha-Col] Abe 
SH5771	<i>S. typhimurium</i>	0-4, 12	[Man-Rha-Gal]  Tyv 
SH4340	<i>S. enteritidis</i>	0-9, 12	[Man-Rha-Gal]  Glc / \
SH5770	<i>S. montevideo</i>	0-6, 7	[Man-Man-Man-Man-GlcNac]

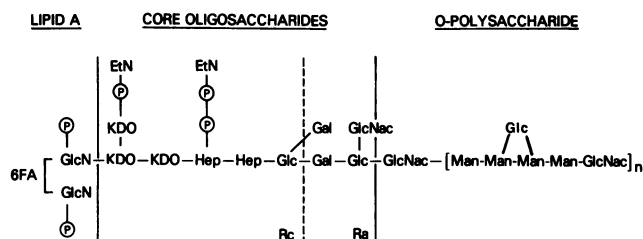


Figure 1. Diagram of LPS of *Salmonella typhimurium* SH5770. *Salmonella* strains are composed of one copy of lipid A, one copy of core oligosaccharide, and from zero to > 60 copies of *O*-polysaccharide subunits. Each *O*-polysaccharide subunit of SH5770 LPS, depicted here, contains four mannose sugar residues. *O*-polysaccharide subunit structures of other strains used in these investigations are shown in Table I. Ra mutants (Ra) of enteric gram negative have a complete lipid A and complete core oligosaccharide, but lack *O*-polysaccharide. Rc mutants (Rc) have a complete lipid A but only part of the core oligosaccharide. Core oligosaccharide structure for *E. coli* J5 and *S. typhimurium* TV119 are not depicted here.

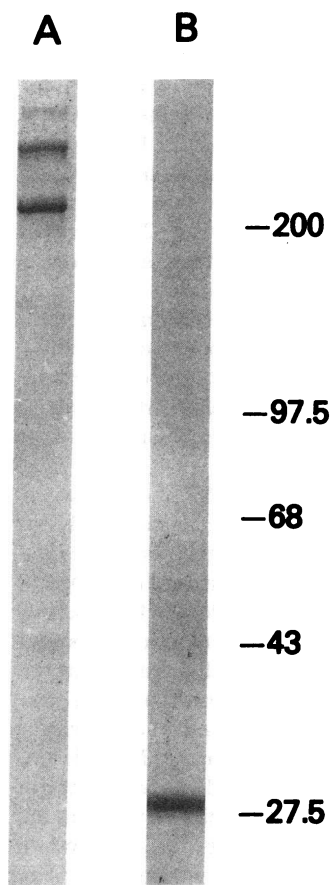
cient serum prepared by the method of Lesavre and colleagues (25) was a gift from Dr. J. Zurlo, National Institutes of Health, Bethesda, MD. Serum deficient in both C1q and factor D was prepared by the method of Praz et al. (26) using preparative chromatography on a Biorex column. Absence of C1q was ascertained by measuring lysis of antibody-sensitized sheep erythrocytes in C1q- and factor D-deficient serum with and without reconstitution of C1q. Factor D deficiency was documented by lysis of rabbit erythrocytes in the deficient serum chelated with Mg-EGTA (see below) with and without added factor D. In each case the serum retained < 2% residual hemolytic activity in the absence of the missing component. In some experiments, serum was heated to 56°C for 30 min to block complement activation or was mixed with an equal volume of HBSS containing 4 mM MgCl<sub>2</sub> and 20 mM EGTA (final concentration 2 mM MgCl<sub>2</sub>, 10 mM EGTA), and heated for 30 min at 30°C to selectively block classical complement pathway activation. To remove naturally occurring antibodies and perhaps MBP from serum, *Salmonella* strains SH5770, SH5771, SH4340, and *S. adelaide* were grown to mid-log phase, then fixed with 0.25% glutaraldehyde. An aliquot of each strain was chilled on ice then used at a concentration of 5 × 10<sup>9</sup> bacteria/ml to adsorb a separate aliquot of ice-cold NHS. Adsorbed sera (ADS) stored at -70°C lost less than 20% of the starting hemolytic titer of C3.

**Antisera.** Antibodies reactive with SH5770 were raised in rabbits according to described procedures (27). A rabbit anti-human MBP antiserum was used as previously described (15).

**Mannose-binding protein.** MBP was prepared as described previously (15). The purified protein was characterized by electrophoresis on a 5-20% gradient SDS-polyacrylamide Laemmli gel (28). Under nonreducing conditions, Coomassie blue staining showed MBP migrated as multimers of ≥ 200 kD (Fig. 2 A). In reducing conditions, MBP migrated as a single band at ~ 30 kD (Fig. 2 B), consistent with its known subunit structure.

For some experiments MBP was derivatized with biotin-*N*-hydroxysuccinimide ester (Behring Diagnostics, La Jolla, CA) suspended in *N,N*-dimethylformamide (Sigma Chemical Co., St. Louis, MO) at 10 mg/ml, according to manufacturer's directions. Briefly, the MBP solution was adjusted to pH 8.5 with 0.01 N NaOH. Biotin was added dropwise to MBP with frequent vortexing, then the mixture was incubated at 25°C for 4 h. The mixture was dialyzed extensively against HBSS 20/1 before use.

After bacteria were incubated in biotinylated MBP then in ADS containing <sup>125</sup>I-C3, MBP was recovered by affinity absorption with Sepharose-avidin (Pierce Chemical Co., Rockford, IL). Samples were first solubilized in 1% SDS, heated at 100°C for 10 min, then diluted with 2 vol of 0.01 M Tris buffer, pH 8, containing 0.25 M NaCl, 0.03% SDS, 1% Triton X-100 and 2 μM NPGb. Then supernatants of sam-



**Figure 2.** Characterization of mannose-binding protein by SDS polyacrylamide gel electrophoresis. Purified MBP was electrophoresed on a 5–10% polyacrylamide Laemmli gel. MBP characteristically migrates as high molecular weight multimers when electrophoresed under non-reducing conditions (lane A), and as monomers of 28–30 kD under reducing conditions (lane B). No other protein bands are seen in the MBP preparation.

ples precleared with Sepharose 4B were incubated overnight at 25°C with tumbling. The resin was then washed five times with the above Tris buffer, and  $^{125}\text{I}$ -C3 bound to the resin counted in a gamma scintillation counter (Packard Multi-Priar; Packard Instrument Co., Inc., Downers Grove, IL).

**Preparation of purified complement components.** Purified C1q, C1r, and C1s were prepared using the methods of Tenner et al. (29), Ziccardi and Cooper (30), and Valet and Cooper (31), respectively. When added to C1q-deficient serum, C1q completely restored classical pathway hemolytic activity. C1s was iodinated in the presence of 5 mM  $\text{CaCl}_2$  using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) as previously described (32). Functionally active human C3 was prepared by the procedure of Hammer et al. (33) with minor modifications, and was radiolabeled with  $^{125}\text{I}$ -Na using Iodobeads (Pierce Chemical Co.) according to manufacturer's instructions. Human factor D (25  $\mu\text{g}/\text{ml}$  in isotonic VBS, pH 7.4) was purified by a modification of the method of Volanakis et al. (34). Factor B (4.3 mg/ml) was prepared using a minor modification of the method of Hammer et al. (33). Just before use, factor B was diluted 1:10 in VBS 5 mM  $\text{CaCl}_2$ , pH 7.5. Guinea pig C2 was purchased from Cordis Laboratories, Miami, FL.

C3 nephritic factor was a gift from Dr. G. Tsokos, National Institutes of Health, Bethesda, MD. It was used at an IgG concentration of 5  $\mu\text{g}/\text{ml}$  in RPMI (Gibco Laboratories, Grand Island, NY).

**Binding of MBP to bacteria.** MBP was allowed to bind to bacteria as described previously (6). Briefly, 10  $\mu\text{g}/\text{ml}$  MBP was added to bacterial suspensions in HBSS 20/1, and the mixture was incubated for 60 min at 25°C rotating end over end at 12–15 rpm. This step was also performed in 2.5 mM  $\text{CaCl}_2$  and 10  $\mu\text{g}/\text{ml}$  MBP with identical results. In some experiments mannan (final concentration 2 mg/ml; Sigma) in HBSS was added to the MBP incubation step. The samples were then washed in HBSS 0.15/1 and used for experiments as described below. Control tubes were incubated in HBSS 20/1 lacking MBP and treated as above.

**MBP effects on C3 deposition.** Bacterial pellets ( $5 \times 10^8$  organisms) of *S. adelaide* and strains SH5771, SH4340, and SH5770 bearing MBP were suspended in 500  $\mu\text{l}$  of 2.5% serum in HBSS 0.15/1 to which  $^{125}\text{I}$ -C3 ( $\sim 3 \times 10^5$  cpm/ml) had been added. After incubation at 37°C for various times, the amount of radiolabeled C3 bound to bacteria was determined as previously described (39). To determine the form of C3 on the bacterial surface, bacterial pellets were washed twice in HBSS 0.15/1, then solubilized in 1% SDS, boiled for 8 min, and divided in two equal aliquots for SDS-PAGE autoradiography. One aliquot of each sample was treated with methylamine (final concentration  $\sim 26$  mM) in carbonate buffer, pH 11, at 37°C for 60 min to release C3 linked by ester bonds (35). Physiologic pH was restored with 0.5 M HCl before SDS-PAGE.

**Selective blockade of classical or alternative pathway activation.** Classical pathway activation was evaluated in 2.5% factor D-deficient serum and in 2.5% C1q- and factor D-deficient serum reconstituted with C1q, but not factor D. To test the activity of the classical pathway, serum deficient in C1q and factor D was diluted to 5% in HBSS 0.15/1 and reconstituted with 3.5  $\mu\text{g}/\text{ml}$  C1q (5% normal serum levels of 70  $\mu\text{g}/\text{ml}$ , [36]). Bacteria ( $5 \times 10^8$ ) in 250  $\mu\text{l}$  HBSS 0.15/1 containing  $^{125}\text{I}$ -C3 were mixed with 250  $\mu\text{l}$  of C1q-reconstituted serum (5%) to yield final concentrations of 2.5% serum and 2.5% normal serum levels of C1q. We examined alternative pathway activation in 2.5% MgEGTA-chelated serum, 2.5% C2-deficient serum, or 2.5% C1q- and factor D-deficient serum reconstituted with increasing amounts of factor D (0.0625 to 1  $\mu\text{g}/\text{ml}$ ).

In addition, purified alternative pathway components, factor B and factor D, were used with C3 nephritic factor to deposit  $^{125}\text{I}$ -C3 on bacteria using previously described methods (37, 38). Briefly, after preincubation of SH5770 in VBS 20/1 with or without MBP, the bacteria were resuspended in 200  $\mu\text{g}$  C3, 10  $\mu\text{g}$  factor B, 1  $\mu\text{g}$  factor D, and 50  $\mu\text{l}$  C3 nephritic factor, then brought to a volume of 500  $\mu\text{l}$  with VBS 5/0. After incubating at 37°C for 60 min, the organisms were washed once with buffer. C3 deposition was amplified in a two-step procedure. Bacteria were incubated at 30°C for 10 min in 10  $\mu\text{g}$  factor B, and 1  $\mu\text{g}$  factor D. The samples were centrifuged at 12,500 g, then 20  $\mu\text{g}$  C3 and 5  $\mu\text{g}$   $^{125}\text{I}$ -C3 were added, and the mixture was incubated at 30°C for 20 min. These steps were repeated for subsequent amplifications. At the end of each amplification, an aliquot was centrifuged through buffer, washed, and the resulting pellet counted to determine the extent of  $^{125}\text{I}$ -C3 bound. Controls were bacteria amplified in C3, factor B and factor D in the absence of MBP, and bacteria "amplified" in buffer in the presence of MBP.

To further explore the possibility that MBP might substitute for C1q to activate the classical pathway, SH5770, *S. adelaide*, or *E. coli* J5 (all at  $5 \times 10^8$  organisms/ml) were incubated in MBP (10  $\mu\text{g}/\text{ml}$ ) or HBSS 20/1 for 60 min at 25°C, then centrifuged to remove MBP and HBSS 20/1. An aliquot of each organism ( $1 \times 10^7$ ) was mixed with various combinations of purified, nonactivated C1 subunits including  $^{125}\text{I}$ -C1s, and C1-inhibitor as described previously (39). Briefly,  $^{125}\text{I}$ -C1s, C1q (18  $\mu\text{g}$ ), C1r (8.8  $\mu\text{g}$ ), C1s (8  $\mu\text{g}$ ), and C1-inhibitor (27  $\mu\text{g}$ ) were mixed at 0°C with 12.6  $\mu\text{l}$  veronal buffer, 6  $\mu\text{l}$  0.05 M  $\text{CaCl}_2$  (final concentration 1 mM Ca) and brought to a final volume of 300  $\mu\text{l}$  with  $\text{H}_2\text{O}$  containing 1% ovalbumin (Sigma). A duplicate mixture was prepared omitting C1q. Bacteria with or without MBP were incubated in 14  $\mu\text{l}$  of one of the above solutions to yield final concentrations of C1r (0.41  $\mu\text{g}$ ) plus C1s (0.37  $\mu\text{g}$ ); or C1q (0.84  $\mu\text{g}$ ), C1r, plus C1s for each  $1 \times 10^7$  organisms. These mixtures were incubated at 30°C for 10 min, and the reaction stopped by addition of SDS-urea-DTT (32). SDS-PAGE (7.5%) and autoradiography were performed. *E. coli* J5 was the positive control organism. *S. adelaide* was the negative control organism. Bacteria plus C1r and C1s in the absence of MBP and C1q were also negative controls.

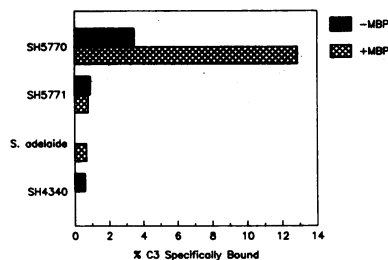
**Serum bactericidal activity.** SH5770 and SH5771 grown to mid-log phase in TSB were washed once then suspended at  $2 \times 10^9$  bacteria/ml (OD 600 = 2.00) in HBSS 20/1. Aliquots were mixed with an equal volume of the same buffer with or without 20  $\mu\text{g}/\text{ml}$  MBP (final concentrations  $1 \times 10^9$  bacteria/ml and 10  $\mu\text{g}/\text{ml}$  MBP). Both tubes were

rotated at 12–15 rpm at 25°C for 60 min. Sample and control mixtures were centrifuged, then pellets were resuspended in HBSS 0.15/1 at a concentration of  $2 \times 10^6$  bacteria/ml. Equal volumes of 2.5, 5, and 10% ADS, MgEGTA-chelated ADS, or HIS were added to the bacteria to obtain final concentration of from 1.25% to 5% serum in HBSS 0.15/1 and  $1 \times 10^6$  bacteria/ml. In some experiments, bacteria were incubated in sub-agglutinating concentrations (1:250 dilution in buffer) of antisera to SH5770. All tubes were then incubated at 37°C, rotating 12–15 rpm for 60 min. Aliquots were serially diluted and plated on GC agar with Isovitalax (Becton Dickinson & Co., Inc., Cockeysville, MD). After overnight incubation at 37°C colonies were counted. Bacterial viability in experimental samples was compared to control tubes containing bacteria incubated in HIS in the presence or absence of MBP.

## Results

**Binding of C3 on bacteria preincubated with MBP.** We tested the hypothesis that MBP would affect C3 deposition on gram negative bacteria containing mannose in the *O*-polysaccharide side chain of LPS. Organisms were incubated under conditions previously shown by Kuhlman et al. (6) to result in MBP binding to organisms bearing mannose-rich LPS. The organisms were then incubated in 2.5% NHS. After presensitization with MBP, strain SH5770 (four mannose sugars in the *O*-polysaccharide repeating unit), bound greater than three and one half times more C3 (Fig. 3) than non-presensitized organisms. More than twice as much C3 was deposited on TV119, the Ra mutant with no side chain but with surface-exposed *N*-acetylglucosamine residues, after MBP incubation (data not shown). SH5771, SH4340, and *S. adelaide* did not bind significantly more C3 after MBP presensitization. Binding of C3 to the Rc mutant, *E. coli* J5, was not affected by MBP (data not shown). Thus there was a positive correlation between the presence of heavily mannosylated LPS or *N*-acetylglucosamine on the bacterial surface and MBP enhancement of C3 binding.

**MBP and C3 binding kinetics.** The kinetics of C3 binding to SH5770 was compared in the presence or absence of MBP. MBP-coated SH5770 rapidly activated complement, reaching half maximal  $^{125}\text{I}$ -C3 binding in < 5 min and peak  $^{125}\text{I}$ -C3 binding in 15–30 min (data not shown). Non-MBP-coated SH5770 reached half maximal  $^{125}\text{I}$ -C3 binding only after 30 min incubation in ADS and peaked after 60 min incubation (data not shown). Binding curves for C3 on SH5771, SH4340 and *S. adelaide* did not differ in ADS whether or not organ-



**Figure 3.** MBP enhancement of C3 binding to strains SH5770, SH5771, SH4340, *S. adelaide*, and TV119. Bacteria were incubated in HBSS 20/1 alone or with MBP 10  $\mu\text{g}/\text{ml}$  for 60 min. Unbound MBP was removed by centrif-

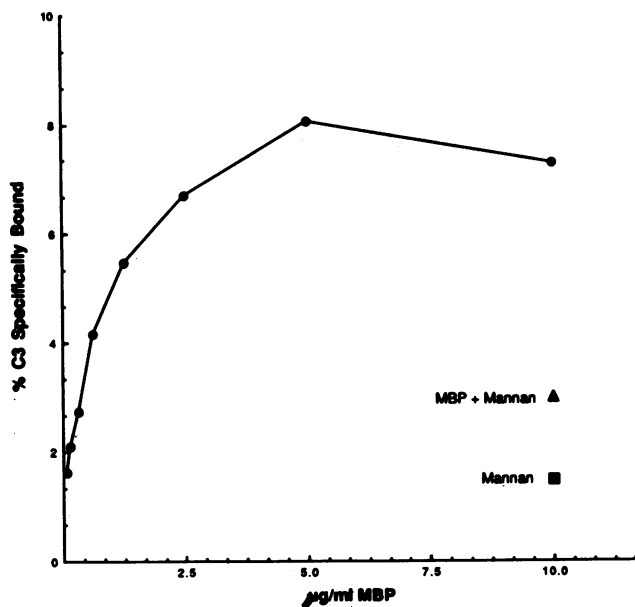
ugation, then the organisms were incubated in 2.5% NHS containing trace amounts of  $^{125}\text{I}$ -C3 at 37°C for 30 min. After centrifugation, radioactivity in supernatants and washed pellets was determined separately. Results are expressed as (cpm bound/total cpm)  $\times$  100. Solid bars represent samples incubated in 20 mM CaCl<sub>2</sub> HBSS alone; cross-hatched bars samples incubated in 10  $\mu\text{g}/\text{ml}$  MBP.

isms were preincubated in MBP. Therefore, for the remainder of experiments presented here, only strain SH5770 was used.

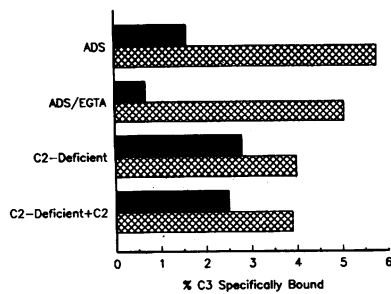
**Dose response to MBP and mannan inhibition.** MBP enhanced complement activation in a dose-related fashion. Concentrations of < 1  $\mu\text{g}/\text{ml}$  of MBP boosted C3 binding on SH5770 (Fig. 4), suggesting that even very small quantities of MBP circulating systemically could be important in the non-immune host. This enhancement is observed in 2.5% serum, implying that MBP might also be important in tissues where quantities of complement components are scarce.

We then sought to competitively inhibit MBP activity with mannan, the complex carbohydrate composed of repeating mannose residues. Mannan added to the mixture of MBP and bacteria during the preincubation step decreased the augmentation of C3 binding by > 60% (Fig. 4). Organisms preincubated in mannan alone were not affected.

**Complement pathway modulated by MBP.** Ikeda et al. recently reported (17) that MBP activates the classical pathway on chromium-treated, mannan-coated sheep erythrocytes. Unexpectedly, in our experiments where classical pathway activation was inhibited by chelation of calcium from serum with EGTA, there was no decrease in percentage of C3 bound to SH5770 (Fig. 5). This finding almost excludes a role for the classical pathway of complement and suggests that C3 deposition results from MBP-mediated activation of the alternative pathway. To confirm and extend this premise, MBP-dependent C3 binding to SH5770 was measured in the absence of components that are critical for classical pathway activation, but are not limiting for the alternative pathway. When SH5770 was incubated with C2-deficient serum (Fig. 5) or C1q- and factor D-deficient serum reconstituted with only



**Figure 4.** Dose response of C3 binding to MBP. SH5770 was incubated in increasing concentrations of MBP (0–10  $\mu\text{g}/\text{ml}$ ), centrifuged to remove unbound MBP, then incubated in 2.5% ADS in HBSS 0.15/1. C3 deposition increased in dose-response fashion. Low concentrations (< 1  $\mu\text{g}/\text{ml}$ ) of MBP boosted C3 binding. When mannan (2 mg/ml) was added to the incubation step with 10  $\mu\text{g}/\text{ml}$  MBP, the amount of C3 bound dropped 60% (solid triangle). Incubation of SH5770 in mannan alone (solid square, no MBP) before serum incubation did not affect C3 deposition.



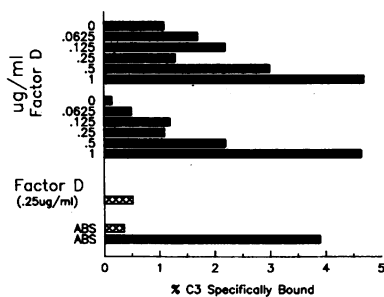
**Figure 5.** Role of the alternative complement pathway in MBP-enhanced complement activation. SH5770 was preincubated in MBP or buffer then in 2.5% serum with both complement pathways intact (ADS or C2-deficient serum reconstituted with C2) or serum with only the alternative pathway intact (ADS/EGTA or C2-deficient serum). Lack of functional classical pathway did not alter MBP enhancement of C3 deposition, nor did reconstitution of the classical pathway in C2-deficient serum further enhance C3 binding.

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factor D (Fig. 6), MBP enhanced C3 deposition substantially. Addition of C2 to C2-deficient serum (Fig. 5) or C1q and factor D-deficient serum (Fig. 7), did not augment complement deposition further. In contrast, the percent C3 bound to MBP-coated organisms was enhanced in a dose-dependent fashion by addition of factor D to C1q- and factor D-deficient serum (Fig. 6). In the absence of factor D, addition of C1q did not effect enhanced C3 binding through MBP (Fig. 7). Increasing amounts of MBP added to C1q- and factor D-deficient serum did not restore C3 activation (Fig. 7) without added factor D. Thus, using at least three different conditions, we determined that MBP enhanced C3 deposition on SH5770 via the alternative complement pathway.

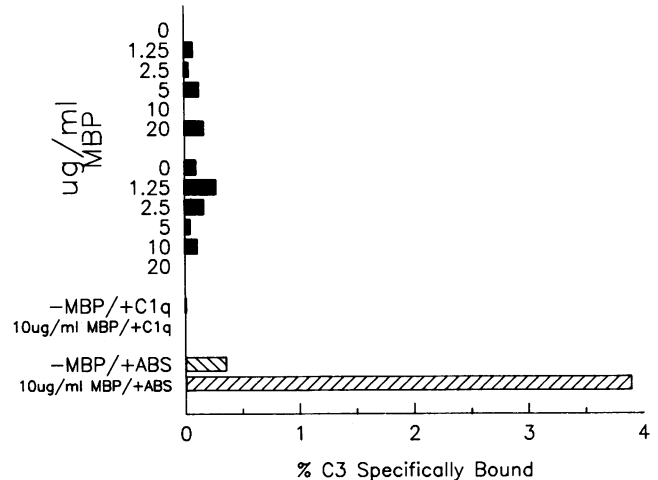
However, we performed the definitive test of alternative pathway mediation of this phenomenon. C3 deposition was tested using purified alternative complement pathway components in the absence of all natural antibodies, all classical pathway components, and all modulators of complement activation. As seen in Fig. 8, SH5770 incubated in purified alternative pathway components in the presence of MBP bound more than twice as much C3 as in the absence of MBP. Clearly, this demonstrated a role for the alternative pathway in MBP-mediated complement deposition.

**Activation of C1 by MBP.** In analogous fashion, the capacity of MBP to activate isolated classical pathway components was tested. Purified C1q, C1r, and C1s were reassociated to



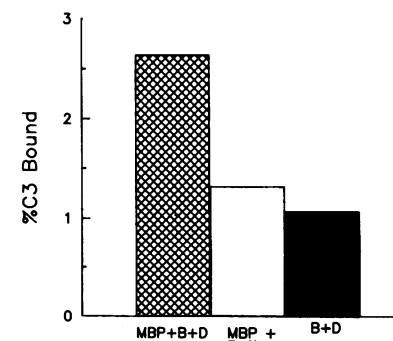
**Figure 6.** Dose response of factor D reconstitution of C1q- and factor D-deficient serum. SH5770 was preincubated in buffer or MBP then in C1q- and factor D-deficient serum. As increasing amounts of factor D were added to C1q- and factor D-deficient serum, enhancement of C3 deposition occurred in a dose-dependent fashion. A control sample was not exposed to MBP, then was incubated in deficient serum to which 0.25  $\mu\text{g}/\text{ml}$  factor D was added (*solitary cross-hatched bar*). Organisms in this sample bound only half as much C3 as organisms preincubated in MBP. The positive control was SH5770 preincubated in buffer or MBP then incubated in ADS. Solid bars indicate samples preincubated in MBP, cross-hatched bars, samples preincubated in buffer. Results of two separate experiments are depicted.

deficient serum, enhancement of C3 deposition occurred in a dose-dependent fashion. A control sample was not exposed to MBP, then was incubated in deficient serum to which 0.25  $\mu\text{g}/\text{ml}$  factor D was added (*solitary cross-hatched bar*). Organisms in this sample bound only half as much C3 as organisms preincubated in MBP. The positive control was SH5770 preincubated in buffer or MBP then incubated in ADS. Solid bars indicate samples preincubated in MBP, cross-hatched bars, samples preincubated in buffer. Results of two separate experiments are depicted.



**Figure 7.** Dose response of C3 deposition to increasing concentrations of MBP. Preincubation of SH5770 in escalating amounts of MBP was followed by incubation in C1q- and factor D-deficient serum. C3 deposition was measured and expressed as in Fig. 2. MBP did not reconstitute classical pathway activity, and no consistent association between concentration of MBP and C3 deposition was observed. Control samples were preincubated in 10  $\mu\text{g}/\text{ml}$  MBP or buffer then incubated in C1q- and factor D-deficient serum reconstituted with C1q or in ADS. Reconstitution of C1q- and factor D-deficient serum with C1q did not result in significant C3 deposition in the presence or absence of MBP. Solid bars represent SH5770 incubated in C1q- and factor D-deficient serum. Slashed bars indicate organisms incubated in ADS. Results of two separate experiments are shown.

form the zymogen C1. *S. adelaide* and SH5770 were preincubated in MBP or buffer then mixed with C1-inhibitor and various combinations of purified, nonactivated, reassociated C1 subunits including  $^{125}\text{I}$ -C1s. *E. coli* J5, which directly activates C1, was used for the positive control. By densitometric scans, 65% of the total radiolabeled C1s was activated by *E. coli* J5 in the presence of C1q, C1r, and C1s, whereas only 13 and 17% activation was observed for *S. adelaide* or SH5770, respectively (Table II, Fig. 9). When these organisms were preincubated in MBP to bind MBP to their surfaces, then incubated with C1r, C1s, and  $^{125}\text{I}$ -C1s in the absence of C1q, only 3% of C1s was cleaved by SH5770 and *S. adelaide*. J5 did not activate C1s at all.



**Figure 8.** MBP enhancement of C3 deposition in purified alternative complement pathway components. SH5770 was preincubated in MBP then incubated in factor B, factor D, C3 nephritic factor and  $^{125}\text{I}$ -C3. After washing, C3 deposition on the organisms was amplified by incubation

in factor B and factor D, and then with  $^{125}\text{I}$ -C3. Twice as much C3 was deposited on SH5770 in the presence of MBP (*cross-hatched bar*) as on organisms preincubated in buffer (*solid bar*). Control organisms preincubated in MBP, subjected to the initial C3 deposition step, but then "amplified" in buffer (*empty bar*) also bound one-half as much C3 as the test sample.

Table II. Activation of C1s by MBP

Organism	C1q, r <sub>2</sub> , s <sub>2</sub>	MBP, C1r <sub>2</sub> , s <sub>2</sub>	C1r <sub>2</sub> , s <sub>2</sub>	MBP, C1q, r <sub>2</sub> , s <sub>2</sub>
<i>E. coli</i> J5	65*	0	0	45
<i>S. adelaide</i>	13	0	0	14
SH5770	17	3	0	4
None <sup>‡</sup>	14	ND	0	ND

\* % C1s activated as determined from densitometric scanning of autoradiograms of samples. These numbers are representative of results of two separate experiments using two different preparations of MBP. C1r used here contained a small amount of activated C1r. In each sample auto-activated C1 was subtracted from sample-activated C1.

<sup>‡</sup> No organism in the reaction mixture.

**Nature of C3 binding to MBP-coated bacteria.** The form of C3 binding to bacterial constituents was compared in the presence and absence of MBP. When bacteria were incubated in serum with an intact alternative pathway, high molecular weight (> 150 kD), covalently bound, C3-acceptor complexes were evident in both the presence and absence of MBP (Figs. 10 and 11 A). Nonactivated C3 migrates in gels as the 120-kD alpha chain and the 75-kD beta chain (Fig. 11). Upon methylamine treatment of MBP-coated bacteria that had been incubated in ADS, MgEGTA-treated ADS (Fig. 11), C2-deficient, or factor D-deficient serum reconstituted with factor D (Fig. 10), C3 was released from the large complexes and migrated as C3b (110 kD alpha chain) and iC3b (68 kD alpha' chain). The ratio of C3b to iC3b was ~ 1:2 by densitometric scanning. In addition, a portion of the bonds formed between C3 and bacterial constituents in the presence of alternative pathway competent sera and MBP appeared to be resistant to the effects of

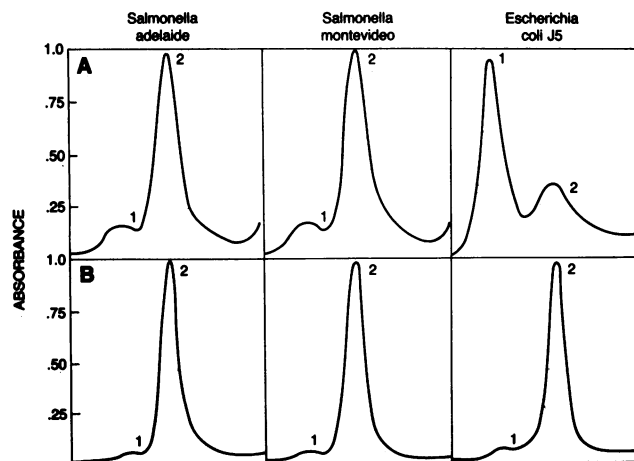


Figure 9. Densitometric scans of autoradiograms of C1s. *S. adelaide*, *S. montevideo*, or *E. coli* J5 were incubated in the presence of C1q, r<sub>2</sub>, <sup>125</sup>I-s<sub>2</sub>, (A), or were preincubated in MBP, then in C1r<sub>2</sub>, <sup>125</sup>I-C1s<sub>2</sub>, (B). Solubilized samples were electrophoresed on Laemmli gels which were then used for autoradiography. Peak 1 of the densitometric scan represents the 58-kD fragment of activated C1s. Peak 2 is the nonactivated 85-kD C1s molecule. *E. coli* J5 directly activates C1 in the presence of C1q, r<sub>2</sub>, s<sub>2</sub>, (A). Neither mannose-rich *S. montevideo* nor mannose-free *S. adelaide* activated significant amounts of C1s under these conditions. None of the organisms activated C1s in the presence of MBP, C1r<sub>2</sub>, s<sub>2</sub> but absence of C1q, (B).

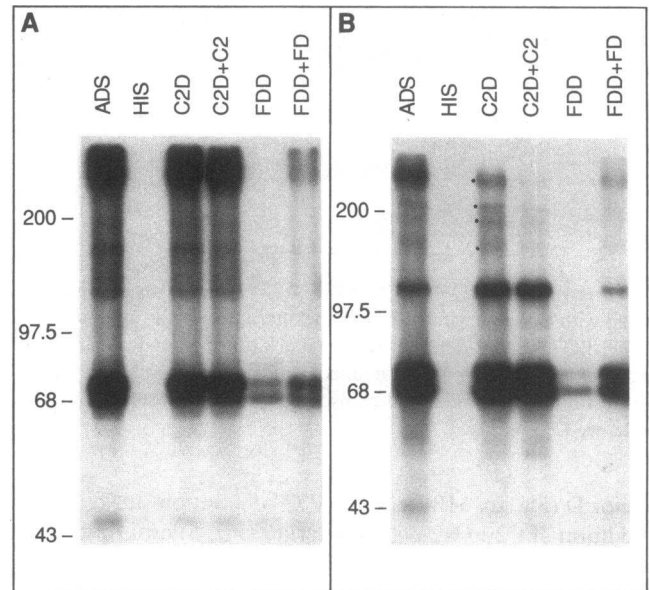
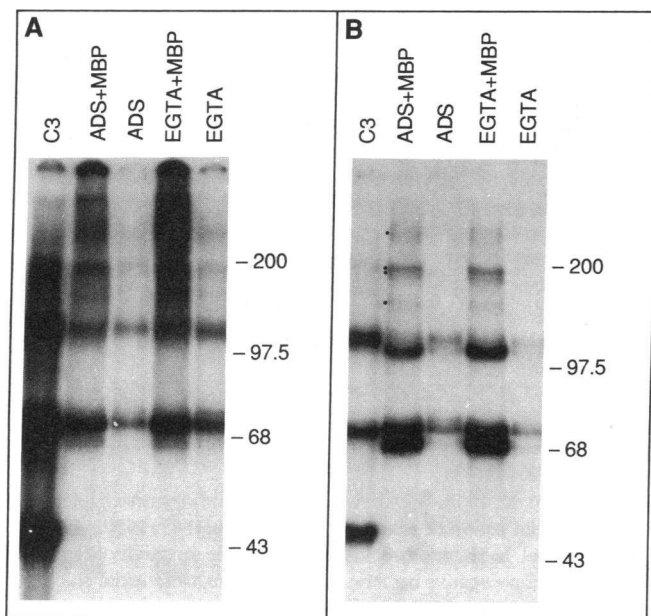


Figure 10. Form of C3 bound to SH5770 and nature of the linkage. All samples of SH5770 are first incubated in MBP, then, as indicated, in ADS, HIS, C2-deficient serum (C2D), C2D reconstituted with C2 (C2D + C2), factor D-deficient serum (FDD), or FDD reconstituted with factor D (FDD + D), all of which had trace amounts of <sup>125</sup>I-C3 added. Autoradiograms of solubilized, washed samples electrophoresed under reducing conditions on 7.5% Laemmli polyacrylamide gels are depicted here. (A) Samples were not methylamine treated. (B) Samples were treated with methylamine before electrophoresis. In each case where the alternative pathway was functional (ADS, C2D, C2D + C2, and FDD + FD) high molecular weight (> 150 kD) C3-acceptor complexes exist (A). Where no alternative pathway is functional (HIS and FDD), large C3-acceptor complexes are absent or greatly reduced. Upon methylamine treatment (B) most of the C3 is released and migrates as C3b and iC3b (~ 1:2 ratio by densitometric scanning). However, in samples containing intact alternative pathways several large complexes persist (extent of migration of complexes indicated by • beside bands), suggesting that some of the C3-acceptor complexes may be linked by amide bonds.

methylamine treatment (Figs. 10 and 11). This result suggests that a portion of the bound C3 in these samples may not be bound by oxy-ester linkages, but rather through amide bonds. In analogous experiments with TV119, some methylamine-resistant, high molecular weight C3-acceptor complexes were apparent in those samples containing MBP-coated TV119 (data not shown). The results suggest that MBP alters the form of C3 bound to gram negative bacteria with surface-exposed ligands and perhaps affects the nature of the bond.

**Biotin-avidin recovery of covalently bound large molecular weight complexes.** We investigated the possibility that C3 might covalently bind to MBP or to a fragment of MBP to form a large molecular weight complex. SH5770 or SH5771 were preincubated in increasing concentrations of biotinylated MBP, then incubated in 2.5% ADS containing <sup>125</sup>I-C3. Organisms were washed and solubilized in 1% SDS at 100°C. We then isolated MBP-<sup>125</sup>I-C3 complexes with avidin-Sepharose. This procedure for affinity purification of C3-MBP complexes in 1% SDS precludes artifactual results due to noncovalent lattice formation between MBP and either LPS or C3 oligosaccharides within C3-acceptor complexes. When MBP was recovered with avidin-Sepharose, < 3% of <sup>125</sup>I-C3 bound to ei-



**Figure 11.** MBP influences the nature of the C3-acceptor linkage. Samples depicted on these autoradiograms are in pairs of SH5770 preincubated with either MBP or buffer, then in serum. In this experiment preincubated samples were incubated in ADS or ADS treated with Mg-EGTA to chelate calcium. (A) Samples were not methylamine treated. (B) Samples were treated with methylamine. Again, released C3 migrates as C3b and iC3b (1:2 ratio). High molecular weight C3-acceptor complexes persist after methylamine treatment in the presence of MBP. The lane marked "C3" contains a portion of the  $^{125}\text{I}$ -C3 added to the serum in these experiments.

ther organism was retained (Table III). Thus there was no evidence suggesting that MBP was a prominent component of the high molecular weight complexes formed when MBP-coated organisms were incubated in serum.

**Bactericidal activity.** Serum bactericidal activity for SH5770 and *S. adelaide* was evaluated. Fig. 12 A shows dose-related killing of SH5770 preincubated in MBP then in increasing concentrations of ADS. In contrast, there was no killing of *S. adelaide* (A) or SH5771 (data not shown) presensitized with MBP.

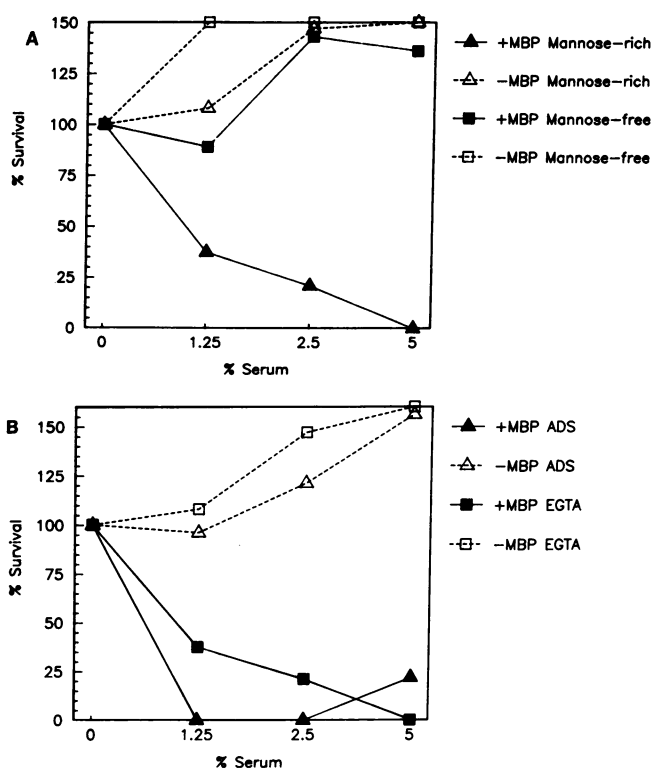
**Table III. Avidin-Sepharose Affinity Absorption of Biotinylated MBP\***

BMBP <sup>‡</sup> μg/ml	% $^{125}\text{I}$ -C3 Recovered	
	Experiment 1	2
8	0.13	3.0
4	0.11	2.6
2	0.38	2.4
1	0	2.1
0	3.5	ND
5 <sup>§</sup>	0.27	0.98

\* The experiment to examine covalent binding of  $^{125}\text{I}$ -C3 to MBP was performed exactly as described in Methods.

<sup>‡</sup> Biotinylated MBP.

<sup>§</sup> μg/ml nonbiotinylated MBP.



**Figure 12.** Effects of MBP on serum bactericidal activity. SH5770 (mannose rich) or *S. adelaide* (mannose free) were incubated in MBP or buffer, then in increasing concentrations of serum (ADS, EGTA, or HIS). Quantitative cultures were performed. Background killing in HIS has been subtracted to depict specific bactericidal activity. SH5770 was susceptible to serum bactericidal activity in the presence but not absence of MBP. *S. adelaide* survived serum bactericidal activity under all conditions tested. Mannose-poor SH5771 (data not shown) also survived whether or not preincubated in MBP.

tized with MBP. Strain SH5770 presensitized with MBP was killed equally well in ADS or EGTA-treated ADS (Fig. 12 B). All strains multiplied in heat-inactivated serum whether or not they were preincubated in MBP. Preincubation in specific antisera similarly enhanced bactericidal activity for SH5770 (data not shown). These results indicate that in addition to enhancing C3 deposition on bacteria with surface mannose residues, MBP also enhances serum bactericidal activity via the alternative complement pathway.

## Discussion

Human MBP interacts with mannose-rich gram negative bacteria to augment complement activation. The effect is mediated through the alternative complement pathway, results in direct bactericidal activity, and occurs at concentrations of MBP that may exist in normal human serum. The effect of MBP is markedly augmented at concentrations of MBP usually achieved during an acute phase response (8). These results suggest that MBP may play a critical role in complement deposition on pathogenic microorganisms in the nonimmune host.

We initially anticipated that MBP would activate the classical complement pathway. MBP was reported to lyse mannan-coated sheep erythrocytes by activating the classical path-



way (17). In addition, MBP is structurally analogous, but bears no actual amino acid sequence homology to the classical complement pathway component C1q (40). Despite structural similarities, MBP was unable to reconstitute the classical pathway (Fig. 7) when C1q was omitted. In addition, neither mannose-rich SH5770 nor mannose-free *S. adelaide* activated significant amounts of C1s in the presence of MBP, C1r, and C1s. We considered briefly the possibility that MBP may activate complement via the unconventional C1-bypass pathway (41, 42). However, MBP-mediated complement fixation occurred in the absence of calcium, and therefore C1-bypass was not considered further. Hence activity tested under the experimental conditions we used can be ascribed to alternative complement pathway activation on bacteria. Interestingly, in some experiments, MBP appeared to inhibit activation of C1s within zymogen C1 (Table II) and therefore it may be an activator of the alternative pathway and inhibitor of the classical pathway. Another acute phase reactant, C-reactive protein, activates the classical pathway and inhibits alternative pathway activation on *Streptococcus pneumoniae* by binding to phosphorylcholine residues in the pneumococcal cell wall (43). Hence, MBP and C-reactive protein may be counter-regulatory acute phase reactant proteins.

MBP-mediated complement deposition on ordinarily serum resistant *Salmonella* strains results in significant bactericidal activity (Fig. 12). Bactericidal activity in the presence of MBP is not simply a consequence of enhanced complement deposition (Joiner, K. A., and J. E. Schweinle, unpublished observations). In this respect, MBP functions analogously to bactericidal IgG for *E. coli* (44, 45) and *Neisseria gonorrhoeae* (39, 46). In contrast to the results with bactericidal IgG and *E. coli* (47), however, covalent complexes between C3 and MBP are not apparently responsible for the enhanced bactericidal activity. Nonetheless, our results indicate that MBP alters the nature of the C3 acceptor bond, resulting in an amide linkage between C3 and putative bacterial acceptor molecules. It is therefore possible that MBP alters the bacterial surface and allows access of C3 to bacterial outer membrane proteins, a possibility we are currently investigating.

In addition to its effects with *S. montevideo* SH5770, MBP also enhanced C3 deposition on the Ra mutant, *S. typhimurium* TV119 and changed a portion of the C3b-acceptor bonds from ester to amide linkages. The possibility was considered that MBP is the bactericidal Ra-reactive factor that binds to *N*-acetyl glucosamine and glycerol-D-mannoheptose residues in LPS of Ra chemotype strains of *Salmonella* and Ra-like strains of other enterobacteria (48, 49). Although the Ra-reactive factor and MBP have several common characteristics, the Ra-reactive factor activates the classical complement pathway (48). We have clearly shown that MBP activates complement by the alternative pathway.

These experiments extend our previous observations showing that MBP can serve as an opsonin independent of complement and immunoglobulins. The current findings emphasize that in the blood stream and at inflammatory sites, MBP and complement molecules probably cooperate in opsonization and clearance of particles by phagocytic cells. It seems likely that each domain of the human MBP has a discrete function. Experiments are in progress to identify the cell attachment regions of MBP and to determine which part of the molecule mediates complement activation.

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