INFECTION AND IMMUNITY, Jan. 1996, p. 380–383 Vol. 64, No. 1
0019-9567/96/$04.00+0

Interactions of Human Mannose-Binding Protein with Lipoteichoic Acids

VSEVOLOD Y. POLOTSKY,[1] WERNER FISCHER,[2] R. ALAN B. EZEKOWITZ,[3] AND KEITH A. JOINER[1]*

Infectious Diseases Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510[1]; Institut für Biochemie, University of Erlangen, D-91054 Erlangen, Germany[2]; and Divisions of Hematology and Infectious Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115[3]

Received 22 June 1995/Returned for modification 18 August 1995/Accepted 19 October 1995

We explored the interaction of human recombinant mannose-binding protein and lipoteichoic acids (LTAs) by enzyme-linked immunosorbent assay. The best ligand was *Micrococcus luteus* lipomannan, followed by *Enterococcus* spp. LTA containing mono-, di-, and oligoglucosyl substituents. LTAs lacking terminal sugars (those of *Streptococcus pyogenes* and *Staphylococcus aureus*) or containing galactosyl substituents (those of *Listeria* spp. and *Lactococcus* spp.) were poor ligands. These results are consistent with known structural requirements for binding through the mannose-binding protein carbohydrate recognition domain.

Human mannose-binding protein (MBP) belongs to a family of defense collagens comprising proteins with short collagen domains which participate in innate pathogen recognition (7, 17, 26). We have recently shown that another member of the defense collagen family, the macrophage scavenger receptor (MSR), binds to gram-positive bacteria and recognizes lipoteichoic acid (LTA) through the collagen region of the receptor (6). The basis for recognition resides in the charge interactions between basic residues in the collagen domain (1, 4, 18) and phosphate groups of LTA (15). Comparison of the collagen domains of MBP and MSR revealed similarity or identity among selected Gly-X-Y repeats (22), especially at the C-terminal portions of the collagen domain, including a cluster of positively charged lysine residues implicated in ligand binding by the MSR (4). This raised the possibility that MBP might bind to LTAs through the collagen domain.

MBP, unlike MSR, is a collectin and contains a carboxy-terminal carbohydrate recognition domain (CRD) adjacent to the collagen domain (7, 26). The CRD of MBP recognizes carbohydrate configurations on a wide variety of pathogens through mannan-inhibitable calcium-dependent binding (8, 16, 19, 21, 24, 27). The CRD interaction with sugar is mediated via direct coordination between a calcium ion and the 3- and 4-hydroxyls of the terminal mannose. Other hexoses that have hydroxyl groups in a similar orientation to those in mannose, such as glucose and GlcNAc are also ligands for MBP, whereas galactose and sialic acid have a different orientation of the 3- and 4-OH groups and lack affinity for MBP (5, 28, 29). A 28-kDa murine protein with similarity to MBP specifically binds to bacterial lipomannan (LM) and to LTAs containing glucosyl and *N*-acetylglucosaminyl substituents (3, 12). These observations suggest that MBP may bind to LTAs through either the CRD or the collagen domain. We have tested both hypotheses and found that MBP interacts with LTAs in accordance with structural predictions for CRD but not those for the collagen domain binding.

* Corresponding author. Mailing address: Infectious Diseases Section, Department of Internal Medicine, Yale University School of Medicine, 808 LCI, P.O. Box 208022, New Haven, CT 06520-8022.

Streptococcus pyogenes II D698 was from the Laboratory of Culture Collection, Institute of Medical Science, Tokyo, Japan; *Listeria monocytogenes* NCTC 7973 and *Listeria welshimeri* SLCC 5334 were from H. Hof, Institut für Medizinische Mikrobiogie und Hygiene, Mannheim, Germany; *Enterococcus faecalis* Kiel 27738 was from G. Hahn, Institut für Hygiene der Bundesanstalt für Milchforschung, Kiel, Germany; *Enterococcus hirae* ATCC 9790 was from G. Shockman, Temple University, Health Science Center, Philadelphia, Pa.; *Staphylococcus aureus* H gol$^-$ Φ^R was from L. J. Douglas, Glasgow, United Kingdom; *S. aureus* DSM 20233, *Enterococcus faecium* DSM 2918, and *Bifidobacterium bifidum* DSM 20239 were from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; *Clostridium innocuum* ATCC 14501 and *Micrococcus luteus* ATCC 4698 were from the American Type Culture Collection, Rockville, Md.; *Lactococcus garvieae* NCDO 2155 was from the National Collection of Dairy Organisms, Reading, United Kingdom; and *Streptococcus pneumoniae* R6 was from R. Hakenbeck, Max-Planck-Inst. Molekulare Genetik, Berlin, Germany.

For preparation of LTAs, bacteria were grown to the late logarithmic phase as described previously (14). LTAs were extracted from mechanically disrupted bacteria with hot phenol-water and purified by hydrophobic-interaction chromatography (9, 14). The purified LTAs and lipoglycans were free of detectable amounts of nucleic acids, polysaccharides, proteins, and cell wall material.

For the binding studies we used 10 poly(glycerophosphate) LTAs (Table 1, 1 to 10) which were analyzed as described previously (10). They differed in substitutions with glycosyl residues and D-alanine ester and varied in the chain length between 19 and 29 glycerophosphate residues per lipid anchor (10–12). Alanine esters were removed by mild alkaline treatment (13) from LTAs 2 and 3 to yield LTAs 7 and 4, respectively (Table 1). LTAs 6 and 8 to 10, lipoglycan 14 and teichoic acid 15 were used in alanine-free forms only. We further included the poly(glycocosyl glycerophosphate) LTAs from *L. garvieae* (11) and *C. innocuum* (12), in which di- and monohexosyl residues are intercalated between the glycerophosphate moieties, the oligo(tetraglycosyl ribostol phosphate) LTA from *S. pneumoniae* (13), a nonglycosylated teichoic acid

TABLE 1. Comparison by ELISA of rMBP binding to LTAs

LTA or lipoglycan no.	Source	Hydrophobic chain structure				% of rMBP-mannan binding[a]
		Description[b]	n[c]	Fatty acid pattern	n_{FA}[d]	
1	*S. aureus*	PGP, S = Ala 0.59, GlcNAc < 0.01[e]	29	br, ss	16.5	8.1 ± 3.0
2	*E. faecalis*	PGP, S = Ala 0.32, Glc_2 0.42[e]	19	ss, su	16.8	14.5 ± 2.7
3	*S. pyogenes*	PGP, S = Ala 0.33	28	ss, su	16.7	9.7 ± 6.4
4	*S. pyogenes*	PGP, Ala free	28	ss, su	16.7	2.3 ± 1.7
5	*S. aureus*	PGP, S = GlcNAc < 0.01[e]	29	br, ss	16.5	6.3 ± 3.5
6	*E. faecium*	PGP, S = Glc 0.23[e], Glc_2 0.3[e]	18	ss, su	16.8	34.2 ± 1.8
7	*E. faecalis*	PGP, S = Glc_2 0.42[e], Ala free	19	ss, su	16.8	34.1 ± 8.0
8	*E. hirae*	PGP, S = Glc 0.32[e], Glc_2 0.17[e], Glc_3 0.02[e], Glc_4 0.34[e]	21	ss, su	17.1	24.2 ± 1.3
9	*L. monocytogenes*	PGP, S = Gal 0.21	26	br, ss	15.8	4.0 ± 1.3
10	*L. welshimeri*	PGP, S = Gal 0.37	25	br, ss	15.9	9.6 ± 9.2
11	*L. garvieae*	Poly(Gal_2Gro*P*) LTA, S = Gal 1.0	11	ss, su	17.1	13.3 ± 0.3
12	*C. innocuum*	Poly(GalGro*P*) LTA, S = GlcN 0.5, GlcNAc 0.25[e]	30	ss, su	17.0	15.6 ± 2.7
13	*S. pneumoniae*	[Glc[e](1→3)-4-amino-6-deoxy-GalNAc(1→4)-6ChoPGal-Nac(1→3)-6-ChoPGalNAc(1→1)-ribitol-5-P]$_n$-glycolipid	6.5	ss, su	16.5	19.0 ± 1.8
14	*B. bifidum*	Lipoglucogalactofuranan	15–25	br, su	17.0	9.3 ± 1.8
15	*S. aureus* H	Teichoic acid, poly(ribitol phosphate)		None	None	17.0 ± 9.6
16	*M. luteus*	LM[e]	56	br, ss	15.2	77.6 ± 2.7

[a] (OD for rMBP bound to LTA/OD for rMBP bound to mannan) × 100. Values are means ± standard errors of the mean.
[b] PGP, Poly(glycerophosphate) LTAs; S, fraction of glycerophosphate residues substituted at position 2′ with the indicated residue. LTA structures are described according to Fischer et al. (10, 11, 14).
[c] n, Length of the hydrophilic chain (for explanation, see text).
[d] n_{FA}, Mean length (number of carbon atoms).
[e] Putative MBP ligand.

(15) and two lipoglycans (14 and 16), the dealanylated glycerophospholipogalactofuranan from *B. bifidum* and the LM from *M. luteus* (23). The chain lengths (n) of compound 11 to 13 represent the numbers of repeats per lipid anchor, and the values for compounds 14 and 16 represent the numbers of hexosyl residues.

The tested species of LTAs and lipoglycans each contained two fatty acids on their glycolipid anchors. As indicated in Table 1, two patterns of fatty acids were observed, one containing branched (br) and straight-chain saturated (ss) fatty acids and the other containing ss and straight-chain monounsaturated (su) fatty acids. The mean length of fatty acids varied between 15 and 17 carbon atoms.

Recombinant MBP (rMBP) was produced as described elsewhere (25, 26). A murine monoclonal antibody with specificity for bound and free MBP (mAb3) (25) was conjugated to horseradish peroxidase and designated αMBP3-HRP. All other reagents were from Sigma (St. Louis, Mo.) unless indicated otherwise.

Dynatech Immulon 2 microtitration plates were coated overnight at 4°C with LTAs or mannan (1 to 100 μg/ml) or with live *S. pyogenes*, *E. faecalis*, and *L. monocytogenes* resuspended to an optical density at 600 nm (OD_{600}) of 0.2 in carbonate buffer (pH 9.55). rMBP binding to ligands was assessed by enzyme-linked immunosorbent assay (ELISA) in the absence or presence of the competitors mannan or poly(5′ guanilic acid) [poly(G)], as described elsewhere (25). The plates were blocked with 5% bovine serum albumin at 37°C for 1 h prior to and after incubation with rMBP. As the negative controls, either LTAs or rMBP were omitted. The specificity of rMBP binding to LTAs was assured by measuring binding in the presence of either 50 mM Ca^{2+} or 20 mM EDTA. Binding in EDTA was subtracted from binding in Ca^{2+} to give specific Ca^{2+}-dependent binding. In some experiments with inhibitors, a fixed concentration of rMBP was used and results were expressed as percent inhibition, calculated as 100 × (OD_{450} without inhibitor − OD_{450} with inhibitor)/OD_{450} without inhibitor. rMBP binding to live bacteria was measured in OD units per organism. The percentage of bacteria adhering to microtitration plates was determined with bacteria metabolically labeled with [^{35}S]methionine (Amersham, Arlington Heights, Ill.) at 10 μCi/ml for 30 min at 37°C.

By ELISA, we compared rMBP binding to 13 LTAs and two bacterial lipoglycans with binding to yeast mannan (Fig. 1a, Table 1). Having established that binding through the CRD reached saturation at an LTA or mannan concentration of 10 μg/ml (not shown), we used the rMBP ligands at this concentration in all subsequent experiments. In the presence of calcium, binding of rMBP was dose dependent and plateaued at an rMBP concentration of 400 to 800 ng/ml for the majority of tested ligands (Fig. 1a). *M. luteus* LM, rich in terminal mannosyl residues, showed the greatest amount of rMBP binding (Fig. 1a, Table 1). *Enterococcus* spp. LTAs (6, 7, and 8 in Table 1) containing glucosyl substituents along the chain showed 2- to 10-fold greater levels of rMBP binding than did *S. aureus* and *S. pyogenes* LTAs lacking glycosyl substituents. Similarly, binding of rMBP to *Enterococcus* spp. LTAs was two to threefold greater than that to *Listeria* spp., *Lactococcus garvieae*, or *Bifidobacterium bifidum* LTAs containing galactosyl substituents, or *S. aureus* teichoic acid, made up of poly(ribitol phosphate). Cleavage of the terminal alanine, which increases the overall negative charge of the LTA and substantially augments binding to the MSR (15), resulted in a twofold increase of *E. faecalis* LTA binding to rMBP, but it did not affect *S. pyogenes* and *S. aureus* LTA binding (Table 1). Thus, the binding of rMBP to LTA generally followed structural predictions for CRD binding. For binding to the collagen domain, the highest level of binding would be expected for the nonglycosylated, purely negatively charged compounds (4, 5, and 14). There is no relationship to the length of the hydrophilic chain. Also the fatty acid composition and the mean chain length of fatty acids seem to play no role in binding to MBP.

We next compared the involvement of CRD and collagen domain in LTA binding. *M. luteus* LM and *E. faecium* LTA

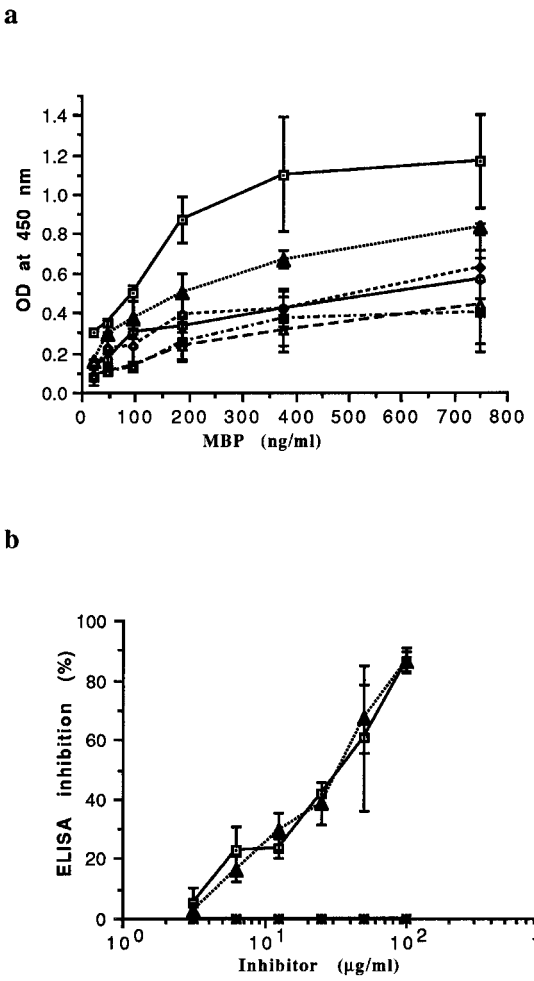

FIG. 1. Comparison by ELISA of rMBP binding to LTAs in the presence of calcium. (a) Binding of increasing concentrations of rMBP to LTAs. The structures of LTAs 3 (■), 5 (◇), 6 (▲), 9 (△), 11 (○), and 16 (⊡) are described in Table 1 according to Fischer et al. (10, 11, 14). (b) Inhibition of rMBP (156 ng/ml) binding to LTAs by mannan or poly(G). Datum points are means ± the standard errors (bars) of the means of three separate experiments. ⊡, *M. luteus* LM-mannan, ▲, *E. faecium* LTA-mannan; ✕, *M. luteus* LM-poly(G); ✚, *E. faecium* LTA-poly(G).

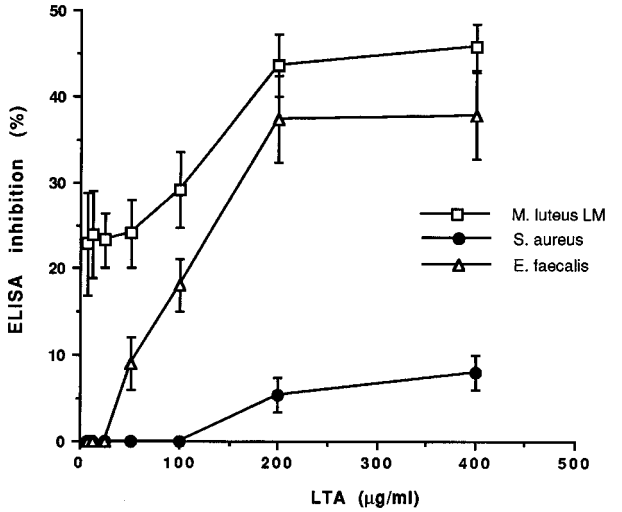

FIG. 2. Inhibition of rMBP binding to mannan by fluid-phase deacylated LTAs. Calcium-dependent binding to mannan was measured by ELISA with rMBP at 156 ng/ml and mannan at 10 μg/ml. Datum points are means ± the standard errors of the means of two separate experiments.

were incubated with a fixed concentration of rMBP (156 ng/ml) in the presence of mannan to compete for CRD binding or in the presence of poly(G) to compete for collagen domain binding (20). With mannan, a dose-response inhibition was observed; 50% inhibition was found with ~40 μg of mannan per ml for both ligands tested (Fig. 1b). The nearly complete inhibition at a mannan concentration of 100 μg/ml, even for poor ligands such as *Listeria* LTAs (not shown), argues strongly for the specificity of the interaction. There was no inhibition of binding with poly(G) at a concentration of 400 μg/ml (Fig. 1b). Also in contrast to the data on MSR binding to LTA (6, 15), we observed no correlation between LTA charge and rMBP binding. These results suggest that the collagen domain is not implicated in rMBP-LTA interaction. In addition, they demonstrate that the collagen domains of MBP and MSR have different recognition functions.

The ELISA results obtained with LTAs 2, 12, and 13 appeared primarily not to correlate well with theoretical predictions for CRD binding. The low rMBP binding to LTAs 2 and 12 may be explained by the presence of positively charged D-alanine ester and positively charged glucosaminyl residues, respectively (Table 1). These residues alternate with the Glc and GlcNAc residues along the chain and therefore might interfere with the calcium-mediated binding of the ligands. Likewise, a low level of binding may be expected for the LTA of *S. pneumoniae*, because it contains only one terminal glucosyl residue and this is linked to the positively charged 2-acetamido-4-amino-2,4,6-trideoxygalactosyl residue (2).

In theory, experiments with LTAs as fluid-phase inhibitors of rMBP binding to mannan or whole bacteria would perfectly supplement our study. However, reevaluation of the data obtained with the 28-kDa mouse serum protein (3) suggested that these experiments may yield erratic results (12). We have made the similar observation for results with rMBP (22). The inconsistency of results from LTA fluid-phase inhibition assays is likely explained by differing aggregate forms of LTAs in solutions (12). The use of deacylated LTA circumvented this problem (Fig. 2). Consistent with results for rMPB binding to solid-phase LTAs (Table 1) as well as with theoretical predictions for CRD binding, deacylated *M. luteus* LM was the best inhibitor of rMBP binding to mannan-coated plates. Deacylated *E. faecalis* LTA also inhibited binding significantly, whereas deacylated *S. aureus* LTA minimally inhibited the rMBP-mannan interaction.

Overall, our data indicate that MBP binds to LTAs through the CRD and indirectly suggest that MBP may play a role in bloodstream clearance of selected LTAs.

This research was supported by grants 5-R01-AI30286 (K. A. Joiner) and 2M01-RR-02172 (R. A. B. Ezekowitz) from the National Institutes of Health, by a grant-in-aid from Bristol-Myers Squibb Institute (R. A. B. Ezekowitz), by a grant from the Pediatric AIDS Foundation (R. A. B. Ezekowitz), and by grant Fi 218/4-8 from the Deutsche Forschungsgemeinschaft (W. Fischer).

REFERENCES

1. **Acton, S., D. Resnick, M. Freeman, Y. Ekkel, J. Ashkenas, and M. Krieger.** 1993. The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands. J. Biol. Chem. **268:**3530–3537.
2. **Behr, T., W. Fischer, J. Peter-Katalinic, and H. Egge.** 1992. The structure of pneumococcal lipoteichoic acid. Improved preparation, chemical and mass spectrometric studies. Eur. J. Biochem. **207:**1063–1075.

3. **Brade, L., H. Brade, and W. Fischer.** 1990. A 28 kDa protein of normal mouse serum binds lipopolysaccharide of gram-negative and lipoteichoic acids of gram-positive bacteria. Microb. Pathog. **9:**355–362.
4. **Doi, T., K. Higashino, Y. Kurihara, Y. Wada, T. Miyazaki, H. Nakamura, S. Uesugi, T. Imanishi, Y. Kawabe, H. Itakura, Y. Yazaki, A. Mutsimoto, and T. Kodama.** 1993. Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors. J. Biol. Chem. **268:**2126–2133.
5. **Drickamer, K.** 1992. Engineering galactose-binding activity into a C-type mannose-binding protein. Nature (London) **360:**183–186.
6. **Dunne, D. W., D. Resnik, J. Greenberg, M. Krieger, and K. A. Joiner.** 1994. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc. Natl. Acad. Sci. USA **91:**1863–1867.
7. **Ezekowitz, R. A. B., L. E. Day, and G. A. Herman.** 1988. A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. J. Exp. Med. **167:**1034–1046.
8. **Ezekowitz, R. A. B., M. Kuhlman, J. Groopman, and R. Byrn.** 1988. A human serum mannose-binding protein inhibits *in vitro* infection by the human immunodeficiency virus. J. Exp. Med. **169:**185–186.
9. **Fischer, W.** 1987. Lipoteichoic acid of *Bifidobacterium bifidum* subspecies *pennsylvanicum* DSM 20239. A lipoglycan with monoglycerophosphate side chains. Eur. J. Biochem. **165:**639–646.
10. **Fischer, W.** 1990. Bacterial phosphoglycolipids and lipoteichoic acids. Handb. Lipid Res. **6:**123–234.
11. **Fischer, W.** 1994. Lipoteichoic acid and lipoglycans. New Compr. Biochem. **27:**199–215.
12. **Fischer, W.** Biological activities of lipoteichoic acids in the mammalian host. *In* E. Faist (ed.), The immune consequences of trauma, shock and sepsis, in press. Springer Verlag, Berlin.
13. **Fischer, W., H. U. Koch, P. Rösel, and F. Fiedler.** 1980. Alanine ester-containing native lipoteichoic acids do not act as lipoteichoic acid carrier. J. Biol. Chem. **255:**4557–4562.
14. **Fischer, W., T. Mannsfeld, and G. Hagen.** 1990. On basic structure of poly(glycerophosphate) lipoteichoic acids. Biochem. Cell Biol. **68:**33–43.
15. **Greenberg, J., W. Fischer, and K. Joiner.** Submitted for publication.
16. **Hartshorn, K., K. Sastry, M. R. White, E. M. Anders, M. Super, R. A. B. Ezekowitz, and A. I. Tauber.** 1993. Human mannose-binding protein functions as an opsonin for influenza A viruses. J. Clin. Invest. **91:**1414–1420.
17. **Holmskov, U., R. Malhotra, R. B. Sim, and J. C. Jensenius.** 1994. Collectins: collagenous C-type lectins of the innate immune defense system. Immunol. Today **15:**67–74.
18. **Krieger, M., S. Acton, J. Ashkenas, A. Pearson, M. Penman, and D. Resnik.** 1993. Structure, binding properties, and functions of macrophage scavenger receptors. J. Biol. Chem. **268:**4569–4572.
19. **Levitz, S. M., A. Tabuni, and C. Treseler.** 1993. Effect of mannose-binding protein on binding of *Cryptococcus neoformans* to human phagocytes. Infect. Immun. **61:**4891–4893.
20. **Pearson, A. M., A. Rich, and M. Krieger.** 1993. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized, four stranded helices. J. Biol. Chem. **268:**3546–3554.
21. **Polotsky, V. Y., J. T. Belisle, K. Mikusova, R. A. B. Ezekowitz, and K. A. Joiner.** Submitted for publication.
22. **Polotsky, V. Y., and K. A. Joiner.** Unpublished data.
23. **Powell, D. A., M. Duckworth, and J. Baddiley.** 1975. A membrane-associated lipomannan in micrococci. Biochem. J. **151:**387–397.
24. **Schweinle, J. E., R. A. B. Ezekowitz, A. J. Tenner, M. Kuhlman, and K. A. Joiner.** 1989. Human mannose-binding protein activates the alternative complement pathway and enhances serum bactericidal activity on a mannose-rich isolate of salmonella. J. Clin. Invest. **84:**1821–1829.
25. **Schweinle, J. E., M. Nishiyasu, T. Q. Ding, K. Sastry, S. D. Gillies, and R. A. B. Ezekowitz.** 1993. Truncated forms of mannose-binding protein multimerize and bind to mannose-rich *Salmonella montevideo* but fail to activate complement *in vitro*. J. Biol. Chem. **268:**364–370.
26. **Super, M., and R. A. B. Ezekowitz.** 1992. The role of mannose-binding proteins in host defense. Infect. Agents Dis. **1:**194–199.
27. **Super, M., S. D. Gillies, S. Foley, K. Sastry, J. E. Schweinle, V. J. Silverman, and R. A. B. Ezekowitz.** 1992. Distinct and overlapping functions of allelic forms of human mannose binding protein. Nat. Genet. **2:**50–55.
28. **Weiss, W. I., G. V. Crichlow, H. M. K. Murthy, W. A. Hendrickson, and K. Drickamer.** 1991. Physical characterization and crystallization of the carbohydrate-recognition domain of a mannose-binding protein from rat. J. Biol. Chem. **266:**20678–20686.
29. **Weiss, W. I., K. Drickamer, and W. A. Hendrickson.** 1992. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature (London) **360:**127–134.

Editor: V. A. Fischetti