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Russell Wallis

Individuals heterozygous for mutant alleles encoding serum mannose-binding protein (MBP, also known as mannose-binding lectin) show increased susceptibility to infections caused by a wide range of pathogenic microorganisms. To investigate the molecular defects associated with heterozygosity, wild-type rat serum MBP polypeptides (MBP-A: 56% identical in sequence to human MBP) and rat MBP polypeptides containing mutations associated with human immunodeficiency have been coexpressed using a well-characterized mammalian expression system. The resulting proteins are secreted almost exclusively as heterooligomers that are defective in activating the complement cascade. Functional defects are caused by structural changes to the N-terminal collagenous and cysteine-rich domains of MBP, disrupting interactions with associated serine proteases. The dominant effects of the mutations demonstrate how the presence of a single mutant allele gives rise to the molecular defects that lead to the disease phenotype in heterozygous individuals. The Journal of Immunology, 2002, 168: 4553–4558.
Materials and Methods

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All tissue culture media were obtained from Life Technologies (Gaithersburg, MD). Promix cell-labeling mix (~70% [13]S)methionine and 30% [35]S]-labeled cysteine) was purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Nitrolotriatic acid-agarose, Sepharose 6B, and protein molecular mass markers were obtained from Sigma-Aldrich (St. Louis, MO). Immulon 4 microtiter wells were purchased from Dynatech Technologies (Chantilly, VA). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). Anti-hemagglutinin (HA) and anti-c-myc mAbs were purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Production of fragments of MASP-1/3 and MASP-2

The N-terminal three domains of MASP-1 and MASP-2, which reproduce the MBP-binding properties of full-size MASPs, were produced in Chinese hamster ovary cells and were purified on nitrotriacetic acid-agarose as described previously (13). The N-terminal three domains of MASP-1 and MASP-3 are identical (5).

Analytical methods

SDS-PAGE was performed using the method of Laemmli (14). Gel filtration chromatography was conducted on a BioSep-S3000 column (300 × 7.8 mm; Phenomenex, Belmont, CA) as described previously for rat serum MBP (4). The composition of oligomers in each MBP was determined by fitting the gel filtration data to multiple Gaussian curves using Microcal Origin. Data are presented as means ± SE from two independent experiments. Complement-fixation activities of MBPs were determined using mannan-coated erythrocytes as targets, following the protocol described previously (4). Results are presented as means ± SE from two independent assays. Solid-phase competition assays were performed by incubating increasing concentrations of MBPs with immobilized fragments comprising the N-terminal three domains of either MASP-1/3 or MASP-2 using [35]S-labeled MBP as the reporter ligand (13). Results are presented as means ± SE from two independent assays performed in duplicate.

Production and purification of MBPs

Mutations were introduced into the cDNA encoding rat serum MBP by substitution of synthetic double-stranded oligonucleotides for restriction fragments. Standard molecular biology techniques were conducted as described elsewhere (15). High selectivity for galactose was introduced into the CRD of MBP by incorporation of the following changes: Glu185 → Gln, Asn187 → Asp, His192 → Tyr, Gly193 → Tyr, Ser195 → Gly, and insertion of the sequence Gly-Leu-Gly-Gly between residues Ser191 and Gly192. The resulting CRDs have previously been designated QPDWG (16). In some cases, epitope tags were attached to the C termini of MBP polypeptides. Synthetic double-stranded oligonucleotides encoding the sequences Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala, derived from influenza HA and Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu, from the human p62c-myc epitope-tagged proteins on 17.5% polyacrylamide gels under reducing conditions. WT* MBP, containing a HA tag and the homozygous R23C/R23C protein with a c-myc tag, were also loaded on the gels as standards. Duplicate immunoblots were probed with Abs specific for the HA and c-myc epitopes. Radioactivity was quantified using a Phosphor Imager SI from Molecular Dynamics (Sunnyvale, CA) after exposure for at least 18 h. Relative amounts of polypeptides were calculated from the levels of radioactivity normalized to the amounts of radioactivity detected in the corresponding standard. The proportion of WT* polypeptides in preparations of MBP purified from two separate cell lines were 0.40 ± 0.09 for the WT*/R23C protein, 0.52 ± 0.07 for the WT*/G25D protein, and 0.51 ± 0.07 for the WT*/G25E protein. Thus, there is no intracellular selection against MBPs containing mutant polypeptides.

Binding of MBPs on mannosyl-Sepharose and galactose-Sepharose affinity columns

MBPs (0.1 mg) were loaded onto mannosyl-Sepharose and galactose-Sepharose affinity columns (1 ml) in 1 ml of loading buffer (50 mM Tris (pH 7.8) containing 1.25 M NaCl and 25 mM CaCl2). Columns were washed with loading buffer (3 × 1-ml fractions), and protein was eluted in 50 mM Tris (pH 7.8) containing 1.25 M NaCl and 2.5 mM EDTA (3 × 1-ml fractions). Galactose (6 mM) was included in the loading buffer for the mannosyl-Sepharose affinity columns to prevent weak interactions with the CRDs of WT* MBP.

Results

Heterooligomers produced by coexpression of wild-type and mutant MBPs

The first step toward understanding why MBPs produced by heterozygous individuals are functionally defective was to determine whether wild-type and mutant polypeptide chains associate with each other during biosynthesis. To address this question, the CRDs of wild-type chains, but not mutant chains, were modified to confer selectivity to galactose (Fig. 1). This strategy enabled selective purification of homooligomeric wild-type and mutant MBPs by affinity chromatography on galactose-Sepharose or mannosyl-Sepharose columns while heterooligomeric MBPs bound to both columns. Because CRDs are not involved in oligomerization of subunits, the changes were not expected to disrupt assembly of the MBPs.

As expected, wild-type MBP binds to a mannosyl-Sepharose column and can be eluted by chelating the Ca2+ required for binding (Fig. 2). It elutes in the unbound wash fractions from a galactose-Sepharose column, indicating that it does not interact significantly with galactose. In contrast, MBP with galactose-binding specificity...
To assess the ability of heteroligomeric MBPs to heterooligomeric MBPs, dominant effects on complement- fixing activities of the proteins are secreted predominantly as heterooligomers. Thus, proteins must contain both mutant and wild-type polypeptides. Hence, heterozygous for the human disorder are likely to produce heterooligomers in which the numbers of wild-type and mutant chains is different. This value is consistent with the 1.5- to 4-fold reduction in complement-fixing activity observed, suggesting that the primary defect is due to aberrant assembly during biosynthesis, resulting in lower amounts of trimers and tetramers of subunits.

Defective complement fixation in the R23C/R23C protein arises because the smaller MBP oligomers have lower affinities for MASP-2 (18). To compare the WT/R23C protein with its homooligomeric counterpart, the binding affinity for MASP-2 was measured using a fragment consisting of the N-terminal three domains of MASP-2 that reproducibly binds the properties of the full-size protein (9). Because the affinity of the MBP-MASP-1 complex is reduced for wild-type MBP, higher concentrations of heterooligomeric MBPs were required to cause equivalent lysis of the target erythrocytes, demonstrating that the heterooligomers have reduced complement-fixing activities (Fig. 3). The complement-fixing activity of the WT/R23C protein is reduced by 1.5- to 4-fold while the activities of the WT/G25D and WT/G28E proteins are reduced by ~5-fold and over 30-fold, respectively. By comparison, the complement-fixing activities of the homooligomeric R23C/R23C, G25D/G25D, and G28E/G28E proteins are reduced by 10-, 7-, and ~100-fold, respectively. Thus, mutations to just some polypeptide chains lead to dominant phenotypes. Because individuals heterozygous for the human disorder are likely to produce heterooligomeric MBPs, defective complement fixation is expected to be a major cause of immunodeficiency in such patients.

Molecular defects in the WT/R23C protein

Previous studies have revealed that the overall complement-fixing activity of MBP is a function of the distribution of oligomers and the specific activities of each oligomer (4). Wild-type rat MBP comprises covalent oligomers ranging from monomers to tetramers of subunits, of which trimers and tetramers have the highest complement-fixing activities. By contrast, the R23C/R23C protein consists of a lower proportion of dimers, trimers, and tetramers of subunits and higher amounts of single subunits, which leads to a reduction in the overall complement-fixing activity (18). Gel filtration analysis shows that the proportion of MBP trimers and tetramers is also reduced in the WT/R23C protein, although not to the same extent as in the R23C/R23C protein (Fig. 4). The major species consist of covalent oligomers that resemble those of wild-type MBP on SDS-polyacrylamide gels, indicating that the disulfide bonds that link individual polypeptide chains together at the N terminus are not disrupted significantly (Fig. 5). Based on the relative complement-fixing activities of oligomers in wild-type MBP (4), the WT/R23C protein would be expected to have ~50% of the activity of wild-type protein if the defect were due to changes in the relative proportion of oligomers alone. This value is consistent with the 1.5- to 4-fold reduction in complement-fixing activity observed, suggesting that the primary defect is due to aberrant assembly during biosynthesis, resulting in lower amounts of trimers and tetramers of subunits.

Defective complement fixation in the R23C/R23C protein arises because the smaller MBP oligomers have lower affinities for MASP-2 (18). To compare the WT/R23C protein with its homooligomeric counterpart, the binding affinity for MASP-2 was measured using a fragment consisting of the N-terminal three domains of MASP-2 that reproducibly binds the properties of the full-size protein (9). Because the affinity of the MBP-MASP-1 complex is reduced for wild-type MBP, higher concentrations of heterooligomeric MBPs were required to cause equivalent lysis of the target erythrocytes, demonstrating that the heterooligomers have reduced complement-fixing activities (Fig. 3). The complement-fixing activity of the WT/R23C protein is reduced by 1.5- to 4-fold while the activities of the WT/G25D and WT/G28E proteins are reduced by ~5-fold and over 30-fold, respectively. By comparison, the complement-fixing activities of the homooligomeric R23C/R23C, G25D/G25D, and G28E/G28E proteins are reduced by 10-, 7-, and ~100-fold, respectively. Thus, mutations to just some polypeptide chains lead to dominant phenotypes. Because individuals heterozygous for the human disorder are likely to produce heterooligomeric MBPs, defective complement fixation is expected to be a major cause of immunodeficiency in such patients.
The apparent molecular mass of the smallest MBP species was determined covalent structures in MBP oligomers have been described previously (18). Possible arrangements of such MBPs. Proteins were separated under nonreducing conditions on 10% polyacrylamide gels and were detected with Coomassie blue. The migration positions of molecular mass markers and of covalent oligomeric forms of wild-type MBP are shown on the left and right, respectively. The covalent structure of WT* MBP resembles the structure of wild-type protein, demonstrating that changes to the CRDs do not affect oligomerization of subunits. As shown in Fig. 4., oligomers of the glycine mutants elute from a gel filtration column at comparable positions to oligomers of wild-type MBP, implying that they are assembled from trimeric subunits. However, these oligomers are assembled primarily from smaller covalent structures of 110, 45, and 26 kDa that comprise four and two polypeptide species, along with some single polypeptide chains. Possible arrangements of such covalent structures in MBP oligomers have been described previously (18). The apparent molecular mass of the smallest MBP species was determined from the migration position on 17.5% gels using molecular mass markers of 6.5–66 kDa as standards.

Structural defects in the WT/R23C protein can also be accounted for by changes in the distribution of oligomers and probably leads directly to aberrant complement fixation. The molecular defects in the R23C/R23C protein result from the presence of cysteine residues within the collagen-like domain (18). Adventitious disulfide bond formation between cysteine residues is believed to disrupt association of the subunits during biosynthesis. The WT/R23C protein contains more of the larger oligomers than the R23C/R23C protein. Thus, incorporation of a cysteine residue into just some polypeptides of the collagen triple helix is less disruptive to oligomer formation than the presence of a cysteine residue in all three polypeptide chains.

Reduced specific complement-fixing activities of individual oligomers of the G25D/G25D and G28E/G28E proteins reflect perturbation of the MASP-2 binding site caused by local disruption to the collagen-like domain and the adjacent cysteine-rich domain (18). These changes lead to a 4-fold decrease in complement-fixing activity. Comparative structural defects in the cysteine-rich domains are detected in the WT/G25D and WT/G28E proteins, as evidenced by the pattern of bands observed on SDS-polyacrylamide gels, in which the predominant covalent species comprise two and four polypeptide chains (Fig. 5). The covalent structures are also comparable to proteins isolated from the serum of immunodeficient individuals that consist of low molecular mass covalent forms.

A 4-fold decrease in complement-fixing activity due to structural perturbations in the N-terminal domains that disrupt the interaction with MASP-2 combined with the small reduction in complement-fixing activity due to the changes in the distribution of oligomers would account for the overall 5-fold decrease in complement-fixing activity of the WT/G25D protein. However, the WT/G28E protein is a further 8- to 10-fold less active at fixing complement than the WT/G25D protein. This reduction mirrors the 10-fold difference in complement-fixing activities between the G25D/G25D and G28E/G28E proteins. Thus, the additional defect caused by the G28E mutation is also a dominant effect.

<table>
<thead>
<tr>
<th>MBP</th>
<th>Monomer (%)</th>
<th>Dimer (%)</th>
<th>Trimer (%)</th>
<th>Tetramer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>13 ± 2</td>
<td>36 ± 10</td>
<td>22 ± 2</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>WT/R23C</td>
<td>31 ± 1</td>
<td>44 ± 2</td>
<td>14 ± 6</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>WT/G25D</td>
<td>21 ± 3</td>
<td>50 ± 6</td>
<td>14 ± 4</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>WT/G28E</td>
<td>23 ± 3</td>
<td>41 ± 2</td>
<td>35 ± 6</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

Previous studies have shown that reductions in the proportion of trimers and tetramers of MBP subunits account for an ~3-fold decrease in the complement-fixing activities of the G25D/G25D and G28E/G28E proteins (18). Gel filtration analysis reveals that the distribution of oligomers in the WT/G25D and WT/G28E proteins is also perturbed compared with wild-type MBP (Fig. 4), although percentages of trimers and tetramers of subunits are somewhat higher than for the G25D/G25D and G28E/G28E proteins. Changes in the distribution of oligomers alone could only account for a reduction of ~25% in the complement-fixing activities, which is much less than the 5-fold and >30-fold reductions observed. Therefore, individual heterooligomers of the WT/G25D and WT/G28E proteins must also have lower specific complement-fixing activities than corresponding homooligomers of their wild-type counterparts.

Heterooligomic MBPs (Fig. 4). Thus, defective binding to MASP-2 by the WT/R23C protein can also be accounted for by changes in the distribution of oligomers and probably leads directly to aberrant complement fixation.

FIGURE 5. SDS-PAGE of homooligomeric and heterooligomeric MBPs. Proteins were separated under nonreducing conditions on 10% polyacrylamide gels and were detected with Coomassie blue. The migration positions of molecular mass markers and of covalent oligomeric forms of wild-type MBP are shown on the left and right, respectively. The covalent structure of WT* MBP resembles the structure of wild-type protein, demonstrating that changes to the CRDs do not affect oligomerization of subunits. As shown in Fig. 4., oligomers of the glycine mutants elute from a gel filtration column at comparable positions to oligomers of wild-type MBP, implying that they are assembled from trimeric subunits. However, these oligomers are assembled primarily from smaller covalent structures of 110, 45, and 26 kDa that comprise four and two polypeptide species, along with some single polypeptide chains. Possible arrangements of such covalent structures in MBP oligomers have been described previously (18). The apparent molecular mass of the smallest MBP species was determined from the migration position on 17.5% gels using molecular mass markers of 6.5–66 kDa as standards.

![Image](http://www.jimmunol.org/DownloadedFrom/4556DOMINANT_EFFECTS_OF_MUTATIONS_IN_MANNOSE-BINDING_PROTEIN)
As expected, the structural changes in the WT/G25D and WT/G28E proteins lead to defective interactions with MASPs. Indeed, solid-phase competition assays reveal that the affinities of the complexes are broadly comparable to those of complexes between the corresponding homooligomeric mutant MBPs and MASPs. Thus, the dominant nature of the phenotypes implies that replacing a glycine residue at the 25 or 28 position by an acidic residue in just one or two polypeptides of the collagen triple helix is almost as disruptive as when all three polypeptide chains contain the mutation. The mutation at the 28 position affects binding to either MASP-1 or MASP-2 more than the mutation at the 25 position. The simplest explanation to account for differences in affinities and complement-fixing activities between the two glycine mutants is that the MASPs bind closer to the 28 position, near the point at which individual subunits diverge from the central core in the bouquet-like structures.

**Discussion**

The results reported here suggest that inefficient complement fixation resulting from defective interactions with MASPs is likely to be a major cause of immunodeficiency in individuals heterozygous for mutant MBP alleles. On coproduction of wild-type and mutant polypeptides, MBPs are secreted predominantly as heterooligomers that have the same structural and functional defects as homooligomeric mutant proteins.

It has been suggested that immunodeficiency in heterozygous individuals arises because only a small fraction of the MBP oligomers will be normal (19). Thus, only 1 in 8 (1 in 2 × 2 × 2) single subunits, 1 in 64 dimers, 1 in 512 trimers, and 1 in 4096 tetramers will contain exclusively wild-type polypeptides, assuming random association of chains. This hypothesis is entirely consistent with data for the WT/G25D and WT/G28E proteins, because the specific complement-fixing activities of these heterooligomeric MBPs are equivalent to the activities of their homooligomeric mutant counterparts. The complement-fixing activity of the WT/R23C protein is less impaired than would be predicted from the model, implying that the defect results from the presence of more than one mutant polypeptide in an MBP oligomer.

Trimerization of MBP polypeptides is initiated by interactions between the C-terminal CRDs and the α helices that form the neck regions and proceed in a C- to N-terminal direction (20, 21). Because WT/mutant MBPs form trimeric subunits and contain both wild-type and mutant polypeptides in comparable proportions, the mutations do not prevent subunit formation. All three mutations are near the N terminus of MBP and so it is likely that only the N-terminal part of the collagenous domain and the cysteine-rich domain are misfolded.

Reduced complement-fixing activities of the WT/R23C and R23C/R23C proteins can be accounted for by changes in the population of oligomers, resulting in a reduction in the amounts of the larger species, although oligomerization of the WT/R23C protein is less impaired. Previous studies have shown that at least some cysteine 23 residues are disulfide bonded to corresponding residues within individual subunits of the homooligomeric mutant MBPs (18). The disulfide bonds probably disrupt interactions between subunits by distorting the collagen triple helix, thus preventing oligomerization of subunits. In heterooligomeric MBPs, in which just half of the polypeptides contain cysteine 23 residues, only a fraction of subunits are likely to contain aberrant interchain disulfide bonds. This distinction probably accounts for the differences in the distribution of oligomers between the WT/R23C and R23C/R23C proteins.

Each protomer of a MASP dimer must bind to a separate subunit of a MBP oligomer to form a stable complex capable of fixing complement (9). Therefore, disruption of even a single MBP subunit due to the presence of a polypeptide containing a mutation is likely to destabilize a MBP-MASP complex. The dominant phenotype resulting from the G25D and G28E mutations in heterozygous individuals probably arises because almost all of MBPs will be heteroligomers that bind defectively to MASP-2.

Reduction in the affinities of MBP/MASP-2 complexes alone cannot account for the lower complement-fixing activities of the homooligomeric and heterooligomeric MBPs (Table I). Binding of MASP-1/3 is also affected by the mutations and the possibility that MASP-1, MASP-2, and MASP-3 function cooperatively cannot be completely ruled out. Because MASP-2 in complex with MBP autoactivates and can trigger complement activation, it is more probable that MASP-2 activation itself is defective in complexes with mutant MBPs. Defective interactions between MBP and MASP-2 probably prevent efficient zymogen activation, thus precluding complement fixation.

There is a complex relationship between disruption of the structure of the collagenous domain of MBP, formation of larger oligomers, MASP-2 binding, and complement activation. For example, the WT/G25D and WT/R23C proteins bind to MASP-2 with broadly comparable affinities but the complement-fixing activity of the WT/G25D protein is ∼2.5-fold lower than the activity of the WT/R23C protein (Fig. 3). The discrepancy between affinities and complement-fixing activities probably arises because the principal molecular defect is different in these two MBPs. In the WT/R23C protein, reduced complement-fixing activity is due largely to defective oligomerization of subunits, whereas in the WT/G25D protein it is caused by structural changes in the collagenous domain. Although both changes affect MASP-2 binding affinity to comparable extents, the effects on MASP-2 activation must be different.

Serum MBP concentrations are considerably higher in heterozygous individuals than in homozygotes. For example, British Caucasoids heterozygous for the mutation equivalent to G25D in rat MBP have only 4- to 5-fold lower serum MBP levels than wild-type individuals, while trace amounts of MBP could be detected in the serum of homozygotes (22). Data reported here indicate that complement-fixation activities of WT/mutant MBPs are all significantly reduced. Thus, defective complement activation may make a more important contribution to the immunodeficient phenotype of heterozygotes than of homozygotes.

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**References**