Expression of Two Different Antiphagocytic M Proteins by *Streptococcus pyogenes* of the OF+ Lineage

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Expression of Two Different Antiphagocytic M Proteins by Streptococcus pyogenes of the OF\(^+\) Lineage\(^1\)

Anette Thern, Maria Wästfelt, and Gunnar Lindahl\(^2\)

All clinical isolates of Streptococcus pyogenes (group A streptococcus) share the ability to resist phagocytosis and grow in human blood. In many strains, this property is due to the expression of a single antiphagocytic M protein, while other strains express more than one M-like molecule, of which the role in phagocytosis resistance is unclear. In particular, all S. pyogenes strains of the OF\(^+\) lineage, representing approximately half of all isolates, express two M-like proteins, Mrp and Emm, which are immunologically unrelated. These two proteins bind different ligands that have been implicated in phagocytosis resistance: Mrp binds fibrinogen and Emm binds the complement inhibitor C4BP. Using a clinical isolate of the common serotype 22, we created immunologically unrelated. These two proteins bind different ligands that have been implicated in phagocytosis resistance: Mrp binds fibrinogen and Emm binds the complement inhibitor C4BP. Using a clinical isolate of the common serotype 22, we created mutants affected in the mrp and emm genes and characterized them in phagocytosis experiments and by electron microscopy. A double mutant mrp\(^−\)emm\(^−\) showed strongly decreased resistance to phagocytosis, while mrp\(^−\) and emm\(^−\) single mutants grew well in blood. However, optimal growth required the expression of both Mrp and Emm. Experiments in which coagulation was inhibited using the specific thrombin inhibitor, hirudin, rather than heparin, indicated that Emm is more important than Mrp for resistance to phagocytosis. Tuftlike surface structures typical for S. pyogenes were still present in the mrp\(^−\)emm\(^−\) double mutant, but not in a mutant affected in the regulatory gene mga, indicating that the presence of these surface structures is not directly correlated to phagocytosis resistance. Our data imply that OF\(^+\) strains of S. pyogenes express two antiphagocytic M proteins with different ligand-binding properties. The Journal of Immunology, 1998, 160: 860–869.

Streptococcus pyogenes (the group A streptococcus) is a common human pathogen that causes a wide variety of diseases, including infections of the throat and skin and a toxic shock syndrome with high mortality (1). Like many other bacterial pathogens, S. pyogenes expresses surface proteins that show great structural variability between strains, a variability that probably allows the bacteria to avoid the immune system of the host. Based on the M protein typing system developed by Lancefield, strains of S. pyogenes can be divided into >80 different serologic groups (2). The M proteins, which are fibrillar molecules with variable N-terminal sequences, are usually considered to be the major virulence factor of S. pyogenes by virtue of their ability to prevent phagocytosis, which allows the bacteria to grow in whole human blood (2–4).

Strains of S. pyogenes can also be divided into two major lineages, OF\(^+\) and OF\(^−\), based on their ability to produce opacity factor (OF),\(^3\) a secreted protein with apoproteinase activity (5, 6). The role of OF in S. pyogenes infections is not clear, but the ability, or lack of ability, to produce this protein appears to reflect a fundamental difference between strains and is correlated to several other properties, such as M protein structure and expression of Fc receptors (7–10). With regard to molecular mechanisms of disease, it is therefore of considerable interest to compare the properties of OF\(^+\) and OF\(^−\) strains.

For many years, it was assumed that strains of S. pyogenes express a single antiphagocytic member of the M protein family. However, many strains (both OF\(^+\) and OF\(^−\)) are now known to express more than one M-like protein, the biologic role of which is unclear (11). In particular, all strains of the OF\(^+\) lineage have three linked genes encoding members of the M protein family: the mrp, emm, and enn genes (10–13). This triplet of genes has also been found in some OF\(^−\) strains (12, 13), but these OF\(^−\) strains are of rare serotypes (14). Thus, the presence of a triplet of genes encoding the Mrp, Emm, and Enn proteins can be considered to be a characteristic property of OF\(^−\) strains.

Little is known about the biologic function of the Mrp, Emm, and Enn proteins encoded by OF\(^−\) strains, and the contribution of these proteins to phagocytosis resistance is unclear. The Enn protein has not been shown to be expressed on the surface of OF\(^+\) bacteria, and the enn gene is transcribed at a very low level, if at all, making it unlikely that Emm makes any contribution to phagocytosis resistance in these strains (15–17). In contrast, the Mrp and Emm proteins are known to be expressed on the bacterial cell surface (18), suggesting that they may have antiphagocytic function. Interestingly, Mrp and Emm show considerable structural differences (19, 20) and do not cross-react immunologically (21). The ligand-binding properties of Mrp and Emm are also different: Mrp binds fibrinogen (18, 20) and Emm binds the complement inhibitor C4BP (22), ligands that may be of importance for phagocytosis resistance (23–25). In addition, both Mrp and Emm bind Ig-Fc, a property that might contribute to virulence (18, 19, 26, 27).

Surprisingly, recent studies have suggested that the contribution of Mrp and Emm to phagocytosis resistance may vary between different strains (28, 29). However, these studies employed strains that had been passed extensively through mice or in the laboratory, procedures that are known to alter the expression of S. pyogenes.

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\(^3\) Abbreviations used in this paper: OF, opacity factor; C4BP, C4b-binding protein; MF, multiplication factor; THY, Todd-Hewitt broth with 0.2% yeast extract; VBS, veronal-buffered saline.
surface proteins and virulence (3, 30, 31). In fact, some of the strains that were analyzed grew poorly in human blood, unlike fresh clinical isolates of S. pyogenes (3, 32). This situation made it desirable to study phagocytosis resistance in an ordinary OF + clinical isolate of S. pyogenes.

Here, we report a study of a clinical isolate of serotype M22, one of the most common S. pyogenes serotypes in the Western world (14, 33). Like other OF + isolates of S. pyogenes, this strain expresses a fibrinogen-binding Mrp protein and a C4BP-binding Emm protein, and it grows rapidly in blood. The construction of a double mutant lacking both Mrp and Emm, and comparison of this strain with strains deficient in only one of the proteins, allowed us to directly assess the contribution of these two proteins to phagocytosis resistance. The results indicate that each of the Mrp and Emm proteins is sufficient to confer phagocytosis resistance in whole blood, i.e., they are antiphagocytic M proteins according to the classical definition (2, 3).

Materials and Methods

Nomenclature

For historical reasons, the proteins in the M protein family expressed by OF + strains have received many different names. Here, we follow the simplified nomenclature proposed by Whatmore et al. (34), and use the designations mrp, emm and enn for the three genes present in OF + strains. Following standard rules, the corresponding three proteins are designated Mrp, Emm, and Enn. It follows that the protein that was previously referred to as Sir22 (18, 35) is referred to here as Emm22, etc. However, the term “M protein” is reserved for a protein that has been shown to have anti-phagocytic function (2, 3).

Bacterial strains, bacteriophage, plasmids, and media

AL168 is a nonmucoid M22 strain of S. pyogenes, isolated in 1989 from a Swedish patient with acute pharyngitis (18). Following primary isolation, the strain was immediately frozen at −80°C and bacteria from the original ampoules were used for this study. A streptomycin-resistant mutant of AL168 (AL168str-r) was isolated and shown to have the same properties as the parental strain with regard to binding of different ligands and ability to grow in human blood. Strain JR54 and its derivative JR5145, which has a deletion in the structural gene for the M6 protein (36), were obtained from Dr. J. R. Scott (Emory University, Atlanta, GA). A derivative of JR5145 expressing the Emm22 (Sir22) protein was constructed by transformation with plasmid pSir22 (35). The Enterococcus faecalis strain CG110, carrying Tn916 (37), was provided by Dr. D. Clewell (University of Michigan, Ann Arbor, MI). M13 was used as the vector for the sequencing of the mrp22 gene and was propagated in Escherichia coli JM101. Plasmid pJR233 (38) was provided by Dr. J. R. Scott. Strains of S. pyogenes were grown on Todd-Hewitt broth (TH) (Difco, Detroit, MI), which was supplemented with 0.2% yeast extract (THY) for experiments involving transposon mutagenesis, electroporation, and phagocytosis. Sheep blood agar plates and THY plates were used for growth on solid phase media. Sheep blood agar plates containing streptomycin (1 mg/ml) and tetracycline (10 μg/ml). C4BP, purified from human plasma (41), was the kind gift of Dr. Bjo¨rn Dahlba¨ck, Malmo¨ Gen¨eral Hospital, Malmo¨, Sweden. Polyclonal IgG was from Pharmacia (Uppsala, Sweden) and polyclonal serum IgA was from Cappel-Organon Teknika (Turnhout, Belgium). All proteins were of human origin.

Tn916 mutagenesis: isolation of mrp22 and emm22 mutants

Mutagenesis with Tn916 was used in an experiment designed to isolate mutants lacking expression of mrp22, mrp22, or emm22. These mutants were expected to have different ligand-binding properties, allowing the use of appropriate screening procedures. For mutants affected in mrp22, mrp22, or emm22, the expected phenotype was lack of ability to bind both fibrinogen and C4BP, only fibrinogen, or only C4BP, respectively (cf. Fig. 3).

Strain AL168str-r was used as the recipient in conjugation experiments with E. faecalis strain CG110, using a method modified from Rubens et al. (42). Both strains were grown to log phase (A620 = 0.4) in THY and mixed in a donor:recipient ratio of 1:50. For filter mating, 15 ml of this suspension was collected on each of many Sartorious cellulose nitrate filters, which were placed on blood agar plates that were incubated at 37°C in 5% CO2 for 17 h. The bacteria were washed off the filters with TH, centrifuged, and resuspended in 1 ml TH per filter. This suspension was plated on blood agar plates containing streptococmycin (1 mg/ml) and tetracycline (10 μg/ml). Transconjugants, which arose at a frequency of 1.4 × 107, were streaked on new selective plates for analysis of ability to bind radiolabeled fibrinogen or C4BP. After growth, the streaks were transferred to a membrane or a paper (see below) that was blocked twice for 30 min in VBS (10 mM Tris, pH 7.4) containing 0.5% gelatin and 0.15% Tween. The blocked membranes/papers were incubated for 1.5 h at room temperature in 5 ml VBS containing 0.1% gelatin and radiolabeled protein (7 × 106 cpm/ml). The membrane filters were then washed 3 × 5 min in VBS, VBS with 0.05% Tween, and VBS, respectively, dried, and subjected to autoradiography overnight. For work with radiolabeled fibrinogen, nontropolulose membrane filters (0.45 mm; Schleicher and Schuell, Dassel, Germany) were found to work best, but for work with C4BP, ordinary copy machine paper gave the best results.

Among ~10,000 Tn916 mutants analyzed, 6 were found to be deficient for fibrinogen binding. PCR analysis of 1 mutant demonstrated that Tn916 was located in or close to the mrp22 gene, since PCR products were obtained when primers derived from the ends of Tn916 were combined with primers derived from the 3′ end of mrp22 and from the intergenic region between the mrp22 gene and the emm22 gene (Fig. 1, lanes 3 and 4). The insertion site was estimated to be located in the promoter region of mrp22 based on the length of the PCR fragments. Several putative mrp22 mutants were also found among the Tn916 mutants, and PCR analysis showed that one of them had Tn916 inserted in the mrp22 gene (Fig. 1, lanes 1 and 2). The insertion site was estimated to be located in the middle of the mrp22 gene. Southern blot analysis showed that the Tn916 mutants affected in mrp22 and mrp22 had only one copy of the transposon inserted into the chromosome (data not shown).

An emm22 mutant was not found among the Tn916 transconjugants and was constructed through insertional inactivation by homologous recombination, as described below.
Isolation of an emm22 deletion mutant and a mp22-emm22 double mutant

A deletion mutant of AL168str-r was constructed in which the 5' half of the emm22 gene was replaced with a kanamycin resistance cassette. The procedure was based on the use of the shuttle vector pJRS233, in which recombination is temperature sensitive in S. pyogenes, allowing efficient selection of recombinants arising through homologous recombination (38). A derivative of pJRS233 was constructed, as described below, carrying a region of recombinants arising through homologous recombination (38). A deriv-...
gene. The Mrp22 and Emm22 proteins bind human fibrinogen and C4BP, respectively (18, 20, 22, 24). Moreover, Mrp22 and Emm22 are both Ig-binding proteins: Mrp22 binds IgG and Emm22 binds IgA as well as IgG (18–20, 35). As mentioned above, expression of the Enn protein has not been demonstrated in any OF\(^+\) strain. Therefore, this study was focused on the function of the Mrp22 and Emm22 proteins expressed by AL168. The sequence of Mrp22 and Emm22 was identical in all of these Mrp proteins. These data support previous studies indicating that the sequence variability among Mrp proteins is less extensive than among Emm proteins (50, 56).

**Sequence of the Mrp22 protein**

The Mrp22 protein (Fig. 4) has an organization similar to that of previously sequenced Mrp proteins, including a putative 41-residue signal peptide, a central region with three A repeats, and a hydrophobic COOH-terminal region preceded by the wall-anchor- ing LPXTG motif (19, 20, 55).

A comparison of Mrp22 with seven other Mrp proteins for which the complete sequences were available revealed three groups of Mrp sequences, represented by the Mrp22, Mrp49, and Mrp76 proteins (Fig. 4). The NH\(_2\)-terminal sequences of the processed proteins vary substantially between the groups, but are at least 92% identical within each group. In contrast, the COOH-terminal parts, including the central repeat regions, are virtually identical in all of these Mrp proteins. These data support previous studies indicating that the sequence variability among Mrp proteins is less extensive than among Emm proteins (50, 56).

**Isolation of bacterial mutants**

Mutants of strain AL168 deficient in expression of the mrp22 and emm22 genes, and a mutant affected in the regulatory gene mga22, were isolated through Tn916 mutagenesis or through the introduction of a deletion, as described in Materials and Methods (Nomenclature section). Mutants, the structure and location in the chromosome was analyzed by PCR, giving products of the expected sizes (Fig. 1). Since the mga, mrp, and emm genes have been found to be transcribed monocistronically in different strains (15, 17, 28), an insertion in one of these genes was not expected to exert a polar effect on downstream genes.

The mutagenesis with Tn916 was devised to allow the isolation of mutants affected in each of the mga22, mrp22, and emm22 genes, but only mga22 and mrp22 mutants were found. The lack of emm22 mutants after Tn916 mutagenesis may be due to the partial site specificity of Tn916 (57). However, a mutant with a deletion in emm22 could be isolated by homologous recombination, employing a temperature-sensitive shuttle vector.

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### Table 1. Oligonucleotides used for sequencing and characterization of mutants

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Description of Target(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’-CGGAAATCTGTGACAGATGTTATGGTG-3’</td>
<td>mga, EcoRI site added(^b)</td>
</tr>
<tr>
<td>2</td>
<td>5’-CTCAGAAATGCCATTGG-3’</td>
<td>mga(^a)</td>
</tr>
<tr>
<td>3</td>
<td>5’-GAAATCTGAGTCTAAACAAAATCCAAACAAG-3’</td>
<td>mrp, EcoRI site added(^c)</td>
</tr>
<tr>
<td>4</td>
<td>5’-TTGCTCATGATTGACAGTACACAGG-3’</td>
<td>mrp(^a)</td>
</tr>
<tr>
<td>5</td>
<td>5’-AGCTCTGTGACGGTCTTGAG-3’</td>
<td>mrp22-emm22 intergenic region, KpnI site added(^d)</td>
</tr>
<tr>
<td>6</td>
<td>5’-AGGAGCGTCTTGCTATTAG-3’</td>
<td>Left end of Tn916, directed outward</td>
</tr>
<tr>
<td>7</td>
<td>5’-CGTCTGATACAAAGCTCATACAT-3’</td>
<td>Right end of Tn916, directed outward</td>
</tr>
<tr>
<td>8</td>
<td>5’-CTCTCATCTCTCTGCTATTG-3’</td>
<td>Right end of Km(_r) in (\Omega)-Km2, directed outward</td>
</tr>
<tr>
<td>9</td>
<td>5’-ATCCGAAAGCAGTCCATAACTCCT-3’</td>
<td>Left end of Km(_r) in (\Omega)-Km2, directed outward</td>
</tr>
</tbody>
</table>

\(^a\) The positions of primers 1–6 are indicated in Figure 3.

\(^b\) Based on mga4 (40), the sequence of which is almost identical to mga22 (A. Thern, unpublished observation).

\(^c\) Based on the sequence of mrp4 (20).

\(^d\) Based on the sequence of emm4 (16).

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**FIGURE 3.** Schematic model of the chromosomal region encoding members of the M protein family in the OF\(^+\) strain AL168. The Mrp22 and Emm22 proteins are expressed on the bacterial cell surface and bind human plasma proteins, as indicated. The Emm22 protein has previously been referred to as the Sir22 protein (35) (see Materials and Methods (Nomenclature section)). The enn22 gene encodes a member of the M protein family, like the mrp22 and emm22 genes, but is probably silent. The mrp22 gene positively controls the expression of the mrp22 and emm22 genes. The positions of different mutations are indicated. The mutations in mga22 and mrp22 are Tn916 insertions. The mutation in emm22 was generated by replacing the 5’ half of the gene with the \(\Omega\)-Km2 element. The positions of primers used for PCR analysis are indicated (cf Table I). The figure is based on sequence data from different sources (35); this report; A. Thern, unpublished data). Unsequenced regions are indicated by dashed lines. See text for details.
For phagocytosis experiments, it was essential to have available a double mutant affected in both of the \textit{mrp22} and \textit{emm22} genes. Such a strain was derived from the Tn916-induced \textit{mrp22} mutant, by introducing a deletion in the \textit{emm22} gene.

An important property of the strain studied here, AL168, is the ability to produce OF, the synthesis of which is positively controlled by the Mga protein (58). As expected, the \textit{mrp22} and \textit{emm22} single mutants and the \textit{mrp22-emm22} double mutant retained the ability to produce OF, but the \textit{mga22} mutant did not (data not shown).

\textbf{Ligand-binding properties of the mutants}

The properties of the mutants were verified by analysis of their ability to bind human serum proteins (cf. Fig. 3). In the sensitive assay used for this purpose, the binding of radiolabeled C4BP, fibrinogen, IgG, or IgA to whole bacteria was measured (Fig. 5).

Wild-type bacteria showed good binding of all four ligands (Fig. 5A). The \textit{mrp22} mutant did not bind fibrinogen, but bound C4BP, IgA, and IgG at a level comparable with that of the wild-type strain (Fig. 5B). The \textit{emm22} mutant lacked the ability to bind C4BP and IgA, as expected, and showed a reduced ability to bind IgG (Fig. 5C). The rather poor IgG-binding capacity of the \textit{emm22} mutant is consistent with previously reported data, showing that Mrp makes only a limited contribution to the IgG-binding ability of the strain (18). As expected, the \textit{mrp22-emm22} double mutant failed to bind any of the four ligands and had binding properties similar to those of the \textit{mga22} mutant (Fig. 5D and E).

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### Table II. Phagocytosis assays

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Heparin</th>
<th>Hirudin</th>
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<tbody>
<tr>
<td>AL168</td>
<td>210 ± 43</td>
<td>750 ± 209</td>
</tr>
<tr>
<td>AL168mrp&lt;sup&gt;−&lt;/sup&gt;</td>
<td>102 ± 28</td>
<td>243 ± 52</td>
</tr>
<tr>
<td>AL168emm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>128 ± 19</td>
<td>98 ± 36</td>
</tr>
<tr>
<td>AL168mrp&lt;sup&gt;−&lt;/sup&gt;emm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>7.4 ± 2.7</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>AL168emm&lt;sup&gt;−&lt;/sup&gt;mga&lt;sup&gt;−&lt;/sup&gt;</td>
<td>5.6 ± 3.4</td>
<td>9.8 ± 4.7</td>
</tr>
<tr>
<td>JRS145</td>
<td>1.5 ± 0.6</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>−</sup>Growth of different bacterial strains in human blood, with heparin or hirudin as anticoagulant. Results are reported as the factor of increase in titer (MF, multiplication factor) during rotation at 37°C for 3 h and are given as mean ± SEM. The data for heparin and hirudin are based on eight and six experiments, respectively, using three different blood donors. See Materials and Methods for details. Graphic presentation of the data is shown in Figure 6.

The binding assays described above indicate that the ability of strain AL168 to bind C4BP, fibrinogen, IgA, and IgG can be attributed solely to the Mrp22 and Emm22 proteins. Moreover, the data indicate that the emm22 gene was expressed normally in the mrp22 mutant, confirming that the presence of Tn916 in mrp22 did not have a polar effect on the expression of emm22.

As mentioned above, the emm gene has not been shown to be expressed in any OF<sup>+</sup> strain. However, in all OF<sup>−</sup> strains tested, the emm gene encodes an IgA-binding protein that can be expressed in E. coli (15, 32), and a characteristic IgA-binding region is found in the NH<sub>2</sub>-terminal part of these proteins (59, 60), including Emm22 (35). If the Emm22 protein were expressed in the strain studied here, the mrp22-emm22 double mutant would therefore have been expected to bind IgA. However, this mutant completely lacked IgA-binding ability (Fig. 5D).

**Resistance of mutants to phagocytosis**

The role of the Mrp22 and Emm22 proteins in phagocytosis resistance was analyzed in the bactericidal test (45), in which streptococcal strains are analyzed for ability to grow in whole human blood (Fig. 6; Table II). The growth indices of strain AL168, the mrp22 mutant, the emm22 mutant, the mrp22-emm22 double mutant, and the mga22 mutant were compared. The availability of the double mutant allowed a direct comparison of the relative contribution of the Mrp22 and Emm22 proteins to phagocytosis resistance. Strain JRS145, an OF<sup>−</sup> strain that does not grow in human blood due to a deletion in the gene for the M6 protein (36), was included as a negative control. The factor of increase in titer during a 3-h incubation period is referred to as the multiplication factor (MF).

In the standard phagocytosis assay (45), anticoagulation is achieved with heparin. However, heparin not only inhibits coagulation but may also affect the complement system (61, 62), a property that could influence the outcome of phagocytosis experiments (63). Indeed, heparin has been reported to bind several complement components, including C4BP (62, 64). This situation prompted us to perform phagocytosis experiments also with blood containing the anticoagulant hirudin, a specific thrombin inhibitor that does not have any known effect on the complement system (65).

Results from eight different experiments with heparinized blood, using three different blood donors, are summarized in Figure 6A and Table II. The wild-type strain AL168 showed an average MF of 210, corresponding to a generation time of 23 min. Thus, AL168 grew rapidly in blood, like other fresh clinical isolates of *S. pyogenes* (2, 32). The mrp22-emm22 double mutant grew poorly, with an average MF of 7.4, i.e., 28-fold less than the wild-type strain. Similar poor growth was observed for the mrp22 and emm22 mutants had MFs that were only 2-fold lower than for the wild-type strain. The differences in MF between the different strains cannot be explained by a general effect on growth, because all strains grew at similar rates in human plasma. Since the single mutants mrp22 and emm22 grew much better than the double mutant, these data indicate that each of the Mrp22 and Emm22 proteins confers phagocytosis resistance. However, optimal growth appeared to require the expression of both Mrp22 and Emm22.

Data from six different experiments with hirudin as the anticoagulant are summarized in Figure 6B and Table II. The results are in good agreement with those obtained with heparinized blood. Thus, the average MF for the double mutant was 1.2, while it was 243 for the mrp22 mutant and 98 for the
emm22 mutant. These data confirm that each of the Mrp22 and Emm22 proteins have antiphagocytic function.

Although the experiments with heparin and hirudin gave similar results, there were some noteworthy differences. First, the wild-type strain AL168 grew somewhat faster in the experiments with hirudin (MF \( 5^{750} \), corresponding to a generation time of 19 min), possibly due to small technical differences between the two types of experiments. More importantly, the difference between AL168 and the \( \text{mrp22} \) and \( \text{emm22} \) mutants was larger than in heparinized blood. Thus, the MFs of the \( \text{mrp22} \) and \( \text{emm22} \) mutants were reduced by factors of 3 and 8, respectively, as compared with AL168. These results show that both the Mrp22 protein and the Emm22 protein were required for optimal growth in blood with hirudin, as appeared to be the case also in heparinized blood. Moreover, the data suggest that the Emm protein was more important than the Mrp protein under these experimental conditions.

In the experiments with hirudin, the \( \text{mga22} \) mutant unexpectedly had a higher MF than the \( \text{mrp22-emm22} \) double mutant. This result was partially due to a MF value of 32, obtained in one experiment. In any case, the \( \text{mga22} \) mutant still grew poorly, as compared with the \( \text{mrp22} \) and \( \text{emm22} \) single mutants. Moreover, the control strain JRS145 did not grow at all, showing that the increased MF value was not due to a general enhancement of growth in blood with hirudin.

Some of the bacterial mutants used here were generated through the insertion of Tn916, encoding tetracycline resistance. However, antibiotics were not present in the bacterial cultures used for phagocytosis experiments. To exclude that growth of a mutant in blood was due to the appearance of revertants, survivors from several experiments were analyzed for their ligand-binding properties. In all cases tested, the survivors had the expected properties.

**Electron microscopy**

Members of the M protein family form \( \alpha \)-helical coiled-coils that extend from the bacterial cell surface and appear as tuftlike or fibrillar projections in the electron microscope (2, 66). Since the OF\(^+\) strain studied here expresses two different members in the M protein family, the Mrp22 and Emm22 proteins, it was of interest to analyze the surface structure of the different mutants described above. The wild-type strain AL168 had the typical tuftlike surface structures (Fig. 7, A and B). As expected from other studies (21, 66, 67), the \( \text{mga} \) mutant lacked these structures and had a smooth surface (Fig. 7F). Interestingly, not only the single mutants lacking Mrp22 or Emm22, but also the double mutant lacking both Mrp22 and Emm22, still expressed the characteristic surface structures (Fig. 7, C–E). However, comparison of many different bacteria indicated that the double mutant expressed about twofold less tufts...
than the single mutants or the wild-type strain. These results indicate that Mrp22 and Emm22 are not the only mga-controlled molecules that contribute to the tuftlike surface structure of strain AL168.

The surface structure of *S. pyogenes* was also analyzed in an OF− strain of serotype M6, which expresses only one molecule in the M protein family. The wild-type strain JRS4 had the typical structure, whereas strain JRS145, which has a deletion in the *emm6* gene, had a smooth surface (Fig. 7, G and H).

To further analyze the structure of the surface proteins expressed by the OF+ strain AL168, the *emm6* deletion mutant JRS145 was transformed with a plasmid expressing the Emm22 protein. The resulting strain had the typical tuftlike surface structures (Fig. 7I), confirming that Emm22 contributes to these structures in strain AL168. A similar experiment with the Mrp22 protein could not be performed, since cloning of the entire *mrp* gene in a shuttle vector was not possible (20).

### Discussion

We have analyzed phagocytosis resistance in a *S. pyogenes* strain of the common serotype 22. This clinical isolate has all of the properties that characterize OF+ strains, including the expression of two surface molecule that are members of the M protein family: a fibrinogen-binding Mrp protein and a C4BP-binding Emm protein. Importantly, this isolate has not been passed in the laboratory or through mice, procedures that are known to influence the expression of members in the M protein family (3, 30, 31). Therefore, it is likely that results obtained with this strain are representative of OF+ strains that cause clinical disease.

The Mrp and Emm proteins of OF+ strains have previously been characterized with regard to structure and ligand-binding properties (10, 15, 18–20, 22, 26, 34, 56). These studies showed that the Emm proteins have the greatest structural similarity to the known antiphagocytic M proteins of OF− strains, which could be taken as evidence that the Emm protein has antiphagocytic function. On the other hand, Mrp binds fibrinogen (18, 20), a well-known property of *S. pyogenes* that has been implicated in phagocytosis resistance (23, 25, 68).

We analyzed bacterial mutants affected in the *mrp* and *emm* genes for resistance to phagocytosis in whole human blood, using heparin or hirudin as the anticoagulant. The overall conclusions that could be drawn from the different experiments were similar, regardless of the method used to achieve anticoagulation. Both sets of experiments showed that a double mutant lacking both Mrp and Emm grew at least as poorly as a mutant affected in the regulatory gene mga. The simplest explanation for this finding is that Mrp and Emm are the only mga-controlled proteins that contribute to phagocytosis resistance. Most importantly, the two sets of phagocytosis experiments showed that the single mutants lacking either Mrp or Emm still grew well in blood, unlike the double mutant lacking both proteins. This result implies that each of the Mrp and Emm proteins has antiphagocytic activity, i.e., they are M proteins, according to classical definitions (2, 3, 45). However, the two sets of experiments also indicated that optimal growth required the presence of both Mrp and Emm. Indeed, lack of the Emm protein reduced growth considerably in the experiments with hirudin. Since all available data indicate that hirudin is a more specific anticoagulant than heparin (61, 62, 64, 65), the results obtained with this compound may be more representative of the in vivo situation. The results can therefore be interpreted to mean that Emm is more important than Mrp for prevention of phagocytosis in whole human blood.

The conclusion that both Mrp and Emm have antiphagocytic function implies a functional redundancy. This situation is not unique to *S. pyogenes*, since many pathogenic microorganisms have been reported to express more than one protein with similar function. For example, *Listeria monocytogenes* expresses two structurally related surface molecules, members of the internalin family, that promote entry into human hepatocytes. Each of these proteins alone promotes entry, but optimal entry requires both proteins (69). Similarly, the adhesion of nontypable *Haemophilus influenzae* to human epithelial cells is promoted by two structurally related surface proteins, both of which can mediate attachment (70). It seems possible that the expression of several proteins with similar, but not identical, function may allow a pathogen to adapt to a changing environment in an optimal fashion. With regard to OF− strains of *S. pyogenes*, the relative importance of the Mrp and Emm proteins could vary during different stages of the infectious process, possibly reflecting roles other than the ability to inhibit phagocytosis.

Two previous studies have addressed the role of the Mrp and Emm proteins in resistance of *S. pyogenes* to phagocytosis. One of these studies (28) analyzed the ability of single mutants affected in the *mrp* or *emm* gene to grow in heparinized blood, and it was concluded that the role of Mrp and Emm varies between strains. One of the strains included in that study, an OF− strain designated 64/14 expresses surface proteins (including an Emm-related protein) with very atypical binding properties (50, 71), making it difficult to compare this strain with other isolates of *S. pyogenes*, particularly since the strain had been extensively passed through mice with the purpose of changing the expression of surface proteins (72). Three other strains analyzed in that study (28) were Lancefield reference strains that had been passed extensively through mice or in the laboratory. This situation may have contributed to the variable results obtained. However, the general conclusion that Mrp contributes to phagocytosis resistance (28) is supported by the results reported here. Another study (29) analyzed the role of the Emm protein for phagocytosis resistance by introducing the *emm4* gene, cloned from an OF+ strain, into the M-deficient mutant JRS145 (derived from an OF− strain of serotype M6). Although the Emm4 (Arp4) protein was expressed on the bacterial cell surface, it did not confer phagocytosis resistance, implying that expression of this Emm protein was not sufficient to prevent phagocytosis. This result may appear to be at odds with the data reported here. However, recent studies in our laboratory have shown that not even the M5 protein, a known antiphagocytic M protein (4), confers phagocytosis resistance on the M-negative mutant JRS145 (H. Kotarsky, G. Lindahl, and U. Sjöbring, manuscript in preparation). This type of problem was avoided in the experiments reported here, which were performed with mutants derived from a single OF+ strain.

As mentioned in the introduction, some strains in the OF− lineage express more than one member of the M protein family, as is the case in OF+ strains. For example, some strains of serotype 1 express two M-like proteins, and a similar situation has been described in some strains of serotype 5 (73, 74). It remains to be analyzed whether each of the two proteins expressed by these OF− strains has an antiphagocytic function. In this context, it is interesting to note that a report published more than 35 years ago (75) demonstrated the presence of two antiphagocytic M proteins in some OF− strains of the rare serotype 14. At the time, these strains were considered to be unusual exceptions, but the properties of OF− strains now suggest that the expression of two antiphagocytic M proteins is very common.

The mechanism by which M proteins prevent phagocytosis is not known. However, the available evidence suggests that one important function of these proteins is to protect against complement...
attack (76, 77). Interestingly, the mechanism used may be to bind human complement inhibitors. Several OF\textsuperscript{−} strains have been shown to bind the complement inhibitor factor H, and direct binding of factor H to purified M6 protein has been demonstrated (77). We have analyzed several OF\textsuperscript{+} strains and found that they bind factor H poorly, if at all, but the Emm proteins of these strains bind another complement inhibitor, C4BP (22, 24). (K. Berggård and G. Lindahl, manuscript in preparation). Thus, it seems possible that OF\textsuperscript{−} and OF\textsuperscript{+} strains use similar mechanisms to prevent complement attack, although they exploit different host proteins to achieve this result. However, the resistance of OF\textsuperscript{−} strains to phagocytosis cannot be completely explained by the ability of Emm proteins to bind C4BP, since the Mrp protein also has anti-phagocytic function, as shown here. Possibly, the anti-phagocytic function of Mrp is due to its ability to bind fibrinogen (23, 25, 68). In any case, it is interesting to note that neither fibrinogen-binding nor C4BP-binding is absolutely required for phagocytosis resistance, as demonstrated by the ability of mrp and emm single mutants to grow in blood.

From electron microscope analysis, it is well known that M proteins form fibrillar or tuftlike surface structures (2, 66). Our analysis of an OF\textsuperscript{−} strain confirmed that molecules in the M protein family are an important part of these structures, but studies of the double mutant mrp emm showed, surprisingly, that this mutant still expressed the typical surface structures, unlike the mgsa mutant. These data indicate that molecules other than those in the M protein family contribute to the characteristic surface structure of OF\textsuperscript{+} S. pyogenes strains. Moreover, the data imply that the presence of tuftlike surface structures is not directly correlated to phagocytosis resistance in S. pyogenes.

In summary, we have presented evidence that OF\textsuperscript{+} strains of S. pyogenes express two different anti-phagocytic M proteins, Mrp and Emm, both of which are required for optimal resistance to phagocytosis. These data provide a molecular basis for further analysis of pathogenetic mechanisms in OF\textsuperscript{+} strains. In particular, it will now be of interest to analyze how the different ligands that bind to the Mrp and Emm proteins influence their biologic properties.

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