Many previous studies have focused on the surface M proteins of group A streptococci (GAS) as virulence determinants and protective antigens. However, the majority of GAS isolates express M-related protein (Mrp) in addition to M protein, and both have been shown to be required for optimal virulence. In the current study, we evaluated the protective immunogenicity of Mrp to determine its potential as a vaccine component that may broaden the coverage of M protein-based vaccines. Sequence analyses of 33 mrp genes indicated that there are three families of structurally related Mrps (MrpI, MrpII, and MrpIII). N-terminal peptides of Mrps were cloned, expressed, and purified from M type 2 (M2) (MrpI), M4 (MrpII), and M49 (MrpIII) GAS. Rabbit antisera against the Mrps reacted at high titers with the homologous Mrp, as determined by enzyme-linked immunosorbent assay, and promoted bactericidal activity against GAS emm types expressing Mrps within the same family. Mice passively immunized with rabbit antisera against MrpII were protected against challenge infections with M28 GAS. Assays for Mrp antibodies in serum samples from 281 pediatric subjects aged 2 to 16 indicated that the Mrp immune response correlated with increasing age of the subjects. Affinity-purified human Mrp antibodies promoted bactericidal activity against a number of GAS representing different emm types that expressed an Mrp within the same family but showed no activity against emm types expressing an Mrp from a different family. Our results indicate that Mrps have semiconserved N-terminal sequences that contain bactericidal epitopes which are immunogenic in humans. These findings may have direct implications for the development of GAS vaccines.

The majority of clinical isolates of GAS have been predicted to contain mrp genes (8, 12, 15), and Mrp functions as a virulence determinant in concert with protein M (16, 17). Mrp is a member of the M protein family of GAS, which includes Emm, Mrp, and Enn proteins. Some emm types only express Emm protein, while others express Emm, Mrp, and/or Enn (15, 18). When Emm and
Mrp are coexpressed, they both appear to be required for virulence (16). Mrp binds human plasma fibrinogen (16, 19) and IgG (18), which confers resistance to phagocytosis and promotes enhanced growth in human blood. In serotopes that express only Emm protein, protective antibodies were directed against the N-terminal regions of the protein (20). When Emm and Mrp were coexpressed, antibodies against both surface proteins were opsonic (16, 21).

In the present study, we show that the N-terminal regions of Mrp have semiconserved sequences that comprise three structurally and immunologically related families (MrpI, MrpII, and MrpIII). Rabbit antisera against recombinant peptides representing the three Mrp families promoted bactericidal activity against different emm types of GAS expressing an Mrp in the same family. Mice that were passively immunized with rabbit antisera against MrpII were significantly protected against death following intraperitoneal challenge infections with M type 28 (M28) GAS. In addition, we show that Mrps are immunogenic in humans, Mrp antibodies were acquired in an age-related manner, and affinity-purified human Mrp antibodies were bactericidal against heterologous emm types of GAS expressing an Mrp in the same family.

MATERIALS AND METHODS
Sequencing mrp genes. Because mrp genes are located in the same position in GAS chromosomes and all have nearly identical leader sequences, a single set of PCR primers was used to amplify the structural genes (16). Mrp sequences were obtained directly from PCR amplicons or after cloning into plasmids. Sequences for the 5' region of mrp genes, encoding the N-terminal domains of Mrps, were obtained using similar methodology.

Mrp sequence comparisons and clustal analysis. Mrp sequences were analyzed for sequence identities and clustal alignments were created using Geneious (version 6.06; Biomatters, Auckland, New Zealand). Sequences of Mrps from emm types 2, 4, 8, 9, 13, 15, 22, 28, 49, 50, 76, N588.2 (now emm98.1) and Alab49 (now emm53) were obtained from references 22 and 28 and from sequences deposited in GenBank under accession numbers X78482, X78483, X78484, X73159, X75750, X69324, M87831, S75411, AAB06612, CP000056, A39392, YP00067280, and CCG26919 (EMBL). Mrp nucleotide sequences from emm types 101, 122, 100.2, 15, B106, 80, 64, 116, 121, 65Mali, 25, 81.2, 67, 124, 30, 42, 65NA, 123, 52, and 59 were obtained in our laboratory as described above (GenBank accession numbers KM69262 to -77). The phylogenetic tree of Mrp was generated with Geneious software using the neighbor-joining and bootstrap methods.

Cloning, expression, and purification of recombinant Mrp peptides. Based on mrp gene sequences, primers were designed to amplify DNA encoding three different N-terminal polypeptide fragments (Mrp2N, Mrp4N, and Mrp49N) representing one Mrp from each of the three structurally related families (MrpI, MrpII, and MrpIII). The primer sequences used to amplify the 5' mrp gene fragments and the added restriction sites were as follows: for the Mrp2N gene fragment (MrpII), GTAAACATGGA GAGCTTAGTGCCGTATTTGTGCAGTTCTGATCA AATCTTTTT; for the Mrp4N gene fragment (MrpII), CTTGAGATCCGAG ATGCCTGTATCGACGCTAC TACGCTTCTTGTAGATT GCCT; and for the Mrp49N gene fragment (MrpIII), GAATACCTGAC TTAATCTTACGACAGCACTTT TCGCACGTGTGCTTTAG GCCATCATTTT.

The Mrp4N gene amplicon was ligated into pTrcHis and introduced into E. coli Top10, and the Mrp2N and Mrp49N gene amplicons were ligated into pET28, transformed into E. coli C3016, as described in histidine fusion products, and purified by metal affinity chromatography as previously described (16). The recombinant proteins consisted of Mrp2N (93 amino acids [aa] plus 6×His), Mrp4N (83 aa plus 6×His) and Mrp49N (83 aa plus 6×His). SDS-PAGE showed that Mrp2N and Mrp49N migrated with an apparent Mr of approximately 10,000 (10K). Mrp4N had an Mr of 14K. Full-length Mrp4, produced for a previous study (16), was approximately 50K in size.

Immunization of rabbits. Rabbits were immunized intramuscularly with 150 μg of Mrp2N, Mrp4N, or Mrp49N that was conjugated in an equal ratio (wt/wt) to keyhole limpet hemocyanin (KLH) and adsorbed to an equal amount of alum (wt/wt) at time zero, 4 weeks, and 8 weeks. Booster injections were given at 12 weeks, and sera were obtained 2 weeks after the final injection.

ELISA. Rabbit antisera against the three recombinant Mrps were assayed by enzyme-linked immunosorbent assay (ELISA), as previously described (16). Because both the immunizing antigens and the ELISA antigens were purified from extracts of E. coli, the antisera were diluted using an extract of the host E. coli to inhibit nonspecific antibody binding (29).

Serologic groupig of laboratory isolates representing 87 different emm types of GAS was performed by whole-cell ELISA (16) using Mrp rabbit antisera that were diluted 1:200 prior to being incubated with whole streptococci. In addition, affinity-purified human Mrp antibodies were assayed for binding to selected GAS isolates by whole-cell ELISA. Nonspecific immunoglobulin binding to the surface of whole streptococci was blocked by a mixture of 3% pig and 2% goat serum prior to the addition of the test antisera. Preimmune rabbit serum was used as a control for each emm type, and the optical densities (OD) of control samples were subtracted from all values obtained with Mrp antisera. Positive antibody binding was recorded for the MrpN antisera that resulted in a net OD of >0.1.

Bactericidal assays. Opossonophagocytic killing (OPK) assays were performed as previously described (30). Briefly, 0.05 ml of Todd–Hewitt broth containing bacteria was added to 0.1 ml of test serum and 0.35 ml of lightly heparinized nonimmune human blood and the mixture was rotated for 3 h at 37°C. Then, 0.1-ml aliquots of this mixture were added to melted sheep’s blood agar, pour plates were prepared, and viable organisms were counted (CFU) after overnight incubation at 37°C. The results were expressed as percent killing, which was calculated using the following formula: [CFU count after 3 h of growth with preimmune serum] − [CFU count after 3 h of growth with immune serum]/CFU count after 3 h of growth with preimmune serum] × 100. In experiments performed with affinity-purified human Mrp antibodies, the control mixtures contained phosphate-buffered saline (PBS) with 1% bovine serum albumin, which was the diluent for the purified antibodies. Only those assays that resulted in growth of the test strain to at least five generations in the presence of preimmune serum were used to express percent killing in the presence of immune serum.

Mouse protection tests. Groups of 15 mice were passively immunized with 0.5 ml of rabbit antisera against Mrp4N (MrpII family) or normal rabbit serum via the intraperitoneal (i.p.) route. Twenty-four hours after the i.p. injection, the mice were challenged i.p. with 4.4 × 10^6 CFU of a virulent M type 28 (M28) GAS, which expresses MrpII protein. Deaths were recorded for 14 days postchallenge. Animal studies were performed in accordance with protocols approved by the Memphis VA Medical Center Research Service and the University of Tennessee IACUC Committees.

Affinity purification of human Mrp antibodies. Sera from healthy adult volunteers were screened for antibodies against the recombinant Mrp proteins by ELISA. Serum from one volunteer was used for purification of Mrp4N antibodies by affinity chromatography, as previously described (31). Briefly, Mrp-specific antibodies were purified over N-hydroxysuccinimide (NHS)-activated Sepharose (GE Healthcare, Uppsal, Sweden) to which Mrp4N had been covalently linked. Antibodies were eluted using glycine-HCl buffer, pH 2.8, neutralized immediately with 1 M Tris (pH 8.8), and dialyzed against PBS.

Detection of Mrp antibodies in serum from pediatric subjects. Serum samples obtained from children aged 2 to 16 (n = 281) were collected from the clinical laboratory of a local children’s hospital. Only the age of the subject and the date of collection were recorded on the transfer tube. Serum was diluted 1:200, and ELISA was performed using recombinant

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Mrp2N, Mrp4N, and Mrp49N as solid-phase antigens. The OD was recorded as an indication of antibody levels against each Mrp. Serum samples from children aged 1 to 2 were used as negative controls. To assess the age-related acquisition of Mrp antibodies, the OD values obtained from each serum sample against all three Mrps were added together and plotted against age. Pearson’s correlation coefficient was used to determine the significance of antibody level versus age of the subject. All protocols using human samples were approved by the University of Tennessee Institutional Review Board and the Human Studies Sub-Committee of the Veterans Administration Research and Development Committee.

RESULTS

Sequence analysis of M-related proteins. Analysis of the translated sequences of mrp genes from heterologous emm types of GAS indicated that the C-terminal sequences were highly conserved and shared almost 100% identity. However, the N-terminal 80 to 90 amino acids of the mature proteins were semiconserved and could be separated into three structurally related families (Fig. 1 and 2). Within each Mrp family, the amino acid sequence identity was as follows: MrpI, average of 94% and range of 72 to 100%; MrpII, average of 90% and range of 77 to 100%; and MrpIII, average of 90% and range of 74 to 100%. Among the Mrp families, the average amino acid identity within the N-terminal regions was 19.7%, with a range of 13.7 to 40.2%. The sequence identity was highest between MrpI and MrpIII sequences (Fig. 1 and 2). Pileup and clustal analyses clearly depicted three main branches representing each family of Mrps (Fig. 2).

Immunogenicity of MrpN peptides. The purified MrpN proteins were conjugated to KLH, adsorbed to alum, and used to immunize rabbits to produce antisera. Antibody titers were determined by ELISA using the homologous and heterologous Mrps as solid-phase antigens (Table 1). Each immune serum reacted at high titer with the immunizing antigen. Low levels of cross-reactive antibodies were detected between Mrp2N and Mrp49N, but not between Mrp2N and Mrp4N.

FIG 1 Alignment of N-terminal amino acid sequences of 33 Mrps from different M types of GAS (A) and alignment of the three Mrp sequences representing the three structurally related families (B). Numbers indicate the M type from which the Mrp sequence was derived. Highlighting indicates identical or similar amino acids.

FIG 2 Phylogenetic tree analysis of Mrp sequences, indicating three related families based on the amino acid sequences of the N-terminal semiconserved regions.
Mrp49N antisera (Table 1), which is consistent with the presence of shared sequence between the two proteins (Fig. 1).

**Mrp expression by GAS as determined by MrpN antibody binding.** The expression of Mrps by multiple *emm* types of GAS was assessed by whole-cell ELISA using rabbit antisera against the three MrpN peptides (Table 2). Of the 87 *emm* types of GAS tested, 70 (80%) reacted with antisera against one of the three Mrp peptides, of which 64% were MrpII positive, 24% MrpIII positive, and 11% MrpI positive. Six *emm* types reacted only with antisera against the full-length Mrp4, which contained C-terminal sequences shared by all Mrps. Eleven *emm* types resulted in no antibody binding.

**Bactericidal (OPK) activity of Mrp antisera against heterologous *emm* types of GAS.** We selected seven different *emm* types of GAS that expressed Mrps, two each from the MrpI and MrpII families and three from the MrpIII family, and performed indirect bactericidal assays using the immune sera against the homologous MrpN peptides (Fig. 3). The results are displayed as percent killing from two independent experiments using blood from two different donors. The Mrp immune rabbit sera promoted bactericidal activity against Mrp-positive GAS isolates that ranged from 12 to 91% (mean, 51%). Bactericidal activity against the Mrp-negative control GAS ranged from 1 to 17% (mean, 10%). These results indicate that the conservation of amino acid sequences within the Mrp families resulted in antibody binding to the bacterial surface that promoted opsonization, phagocytosis, and killing.

**TABLE 1 Mrp serum antibody titers evoked in rabbits**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Mrp2N (MrpI)</th>
<th>Mrp4N (MrpII)</th>
<th>Mrp49N (MrpIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Anti-Mrp2N</td>
<td>25,600</td>
<td>400</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Anti-Mrp4N</td>
<td>400</td>
<td>25,600</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Anti-Mrp49N</td>
<td>1,600</td>
<td>800</td>
<td>25,600</td>
</tr>
</tbody>
</table>

The Mrp family of the antigen is shown in parentheses.

**TABLE 2 Mrp families of GAS defined by antibody reactivity with whole bacteria**

<table>
<thead>
<tr>
<th>Mrp antibody binding</th>
<th>GAS Emm type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MrpI</td>
<td>2, 65NA, 69, 92, 102, 114, 117, 118</td>
</tr>
<tr>
<td>Anti-MrpII</td>
<td>4, 8, 25, 28, 30, 32, 33, 41, 43, 53, 58, 60, 64, 65Mali, 66, 67, 70, 71, 72, 74, 78, 79, 80, 82, 83, 86, 88, 89, 91, 93, 95, 97, 98, 100, 101, 105, 108, 109, 111, 115, 116, 120, 121, 124</td>
</tr>
<tr>
<td>Anti-MrpIII</td>
<td>9, 11, 22, 44/61, 49, 52, 59, 68, 73, 84, 85, 94, 96, 106, 107, 112, 113</td>
</tr>
<tr>
<td>Anti-Mrp full length</td>
<td>48, 75, 87, 99, 104, 110</td>
</tr>
<tr>
<td>No antibody binding</td>
<td>12, 13, 31, 34, 42, 50, 56, 76, 77, 81, 122</td>
</tr>
</tbody>
</table>

* Binding of antibodies was considered positive if ELISA values were >0.1 after subtracting the background OD observed with preimmune rabbit serum. Average ODs of antisera were as follows: Mrp2N antisera, 0.87 (range, 0.22 to 1.78); Mrp4N antisera, 0.86 (range, 0.19 to 1.52); Mrp49N antisera, 0.49 (range, 0.12 to 0.85).

* Laboratory strain of type *emm*22 reacted with anti-MrpIII antisera, but the GenBank sequence aligned with MrpII.

* *emm* types predicted to be Mrp negative that were tested and resulted in no antibody binding were M3, M6, M14, M18, M19, and M24.

**FIG 3 Bactericidal (OPK) activity of rabbit MrpN antisera against different *emm* types of GAS expressing Mrps in the homologous family.** Black and gray bars represent the percent killing observed in two independent assays with blood from different donors. GAS of M types 3, 18, and 24 served as Mrp-negative controls.

**FIG 4 Kaplan-Meier survival plot of mice (n = 15 per group) that were passively immunized i.p. with rabbit antiserum against Mrp4N (circles) or normal rabbit serum (triangles) and then challenged 24 h later with M28 GAS via the i.p. route. Survival after 14 days was 67% in the Mrp4N group versus 26% in the control group: *P* = 0.046, log rank chi-square test on Kaplan-Meier survival data.**

**Age-related acquisition of human Mrp antibodies.** Serum samples from pediatric subjects ranging in age from 2 to 16 years were screened for the presence of Mrp antibodies by ELISA (Fig. 5). The average OD values from two experiments using a serum dilution of 1:200 were used as indicators of antibody levels against each MrpN protein. To assess the relationship between the age of the subject and overall Mrp antibody levels, the average OD values for each serum sample against all three MrpN proteins were added and plotted against age, which resulted in a correlation coefficient of 0.24 (*P* < 0.001). Serum from subjects age 1 to 2 resulted in an average OD of 0.11 ± 0.06 (standard deviation). Although not all
subjects had Mrp serum antibodies detected, the higher levels were associated with those subjects in the older age groups (Fig. 5).

**Bactericidal (OPK) activity of human Mrp antibodies.** Mrp4N-specific antibodies were affinity purified and tested for binding to the surface of intact bacteria by ELISA and for bactericidal activity against Mrp-positive GAS representing different emm types (Fig. 6). The titer of the purified antibody preparation against Mrp4N (MrpII) was 25,600, and its titers against Mrp2N and Mrp49N were <200. The bactericidal activities of the purified antibody against GAS emm types expressing the homologous MrpII protein ranged from 26 to 81% killing (mean, 50%), whereas the percent killing of GAS expressing an MrpIII protein ranged from 0 to 7% (Fig. 6A). Mrp4N antibody binding to the surface of whole bacteria correlated significantly with functional bactericidal activity (Fig. 6B). Taken together, our results suggest that Mrp is immunogenic in humans, the antibodies are acquired in an age-related manner, and human Mrp antibodies are bactericidal against GAS that express Mrps in the same structurally related family.

**DISCUSSION**

In the present study, we showed that Mrp sequences were semi-conserved and that the N-terminal structures determined to date defined three structurally and serologically distinct families. Antisera raised against N-terminal recombinant peptides representing the three Mrp families were opsonic and promoted various levels of bactericidal activity against emm types of GAS that expressed Mrps of the homologous family. Mice were protected against challenge infections with M28 GAS after passive immunization with rabbit antisera against Mrp4N. These results suggest that epitopes of Mrp could contribute to protection against infection in humans by Mrp-positive GAS.

The identification of protective antigens of GAS has generally followed two different approaches, using (i) M proteins and M peptides, which are variable in sequence, and (ii) common (shared) antigens that evoke protective immune responses against a broad range of serotypes. The identification of protective epitopes contained in the N-terminal regions of semiconserved M-related proteins potentially adds a third approach to GAS vaccine formulation. Mrp has not previously been considered a GAS vaccine antigen candidate. Four large-scale proteomic/reverse vaccinology approaches used by investigators to identify potential protective antigens were based on the genome/proteome of type 1 GAS, which is mrp negative. Therefore, Mrp was not identified as a potential vaccine component by any of these modern vaccinology techniques.

We showed that Mrp is immunogenic in humans and that the acquisition of Mrp antibodies is correlated with increasing age. Affinity-purified human Mrp antibodies were bactericidal against heterologous emm types of GAS expressing Mrps in the same structurally related family. It is well known that GAS infections primarily afflict school age children and that the incidence of infection decreases with age, which has been postulated to be due to an accumulation of immunity against common emm types or against shared GAS antigens following repeated infections with different emm types. The age-dependent acquisition of Mrp antibodies may also play a role in the relative resistance of older individuals to GAS infections.

Mrp is predicted to be expressed by a majority of emm types of...
GAS, especially those prevalent in tropical or subtropical regions of the world with populations at high risk for ARF and RHD. Recent epidemiological studies in Bamako, Mali, indicated that 89% of 305 GAS isolates from children with symptomatic pharyngitis were predicted to be Mrp positive (12). Similarly, 88% of 157 GAS throat isolates from children in the Vanguard community of Cape Town were Mrp positive (13). The percentages of clinical isolates from Mali and Cape Town representing serotypes of GAS not included in the current 30-valent M protein-based vaccine (36) that were Mrp-positive were 96% and 100%, respectively. These observations suggest that Mrp could be an important addition to M protein-based vaccines designed to provide broader potential efficacy.

The levels of bactericidal activity and protective efficacy achieved with Mrp antiserum in this study were generally lower than those observed in previous studies with M protein antiserum (30). This observation may be related to lower levels of Mrp antibody binding to the bacterial surface compared to the binding of M antibody. This could result from lower antibody affinity for slightly different Mrp sequences or, alternatively, to smaller amounts of Mrp being expressed on the bacterial surface of some strains. Both of these possibilities are consistent with our observation demonstrating a direct correlation between human Mrp antibody binding to the cell surface and killing.

The observation that N-terminal Mrp sequences were conserved within each family and yet were variable among families suggests that there has been conservation of structure based on some as-yet unknown function. The structural constraints may also limit the variability of epitopes that would otherwise result from immunological pressure and escape mutations. Nonetheless, the finding that Mrp antibodies were opsonic and Mrp sequences were semi-conserved suggested that as few as three Mrp peptides could potentially add to the protective efficacy and breadth of coverage of multivalent M protein-based vaccines, particularly in areas of the world where ARF and RHD are still very prevalent. We have recently shown that a combination of antiserum against Mrp and M protein promoted phagocytosis to a greater degree than either one alone, which provides additional support for the concept of formulating GAS vaccines that contain both antigens (21). Studies are currently in progress to directly assess the protective immunogenicity of combination vaccines formulated to contain M and Mrp peptides. We believe that such vaccines could provide broader coverage and have a greater impact on the overall burden of GAS infections and their complications.

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J.B.D. is the inventor of certain technologies related to the development of group A streptococcal vaccines. The University of Tennessee Research Foundation has licensed the technology to Vaxent LLC, of which J.B.D. is the Chief Scientific Officer and a member. All other authors have no conflicts.

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