Role of *emm* and *mrp* Genes in the Virulence of Group A Streptococcal Isolate 64/14 in a Mouse Model of Skin Infection

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The virulence of group A streptococcal isolate 64/14 and paired isogenic mutants in which either the *emm* or *mrp* gene had been insertionally inactivated was compared in mice. Loss of expression of the *emm* gene product resulted in a significant loss of virulence when the isolate was injected into the skin but had no significant difference when injected intraperitoneally. By contrast, inactivation of the *mrp* gene caused the organism to be more virulent in the skin, while having no significant effect intraperitoneally. An isogenic mutant, in which the *mga* gene was inactivated and neither the *emm* gene nor the *mrp* gene was expressed, demonstrated no significant difference in virulence from the wild type organism. Organisms recovered from the spleen of mice lethally infected with the *mga* mutant expressed all Mga-regulated IgG-binding gene products despite the presence of the spectinomycin-resistance cassette, which was used to inactivate the *mga* gene, in its original position.

Group A streptococci continue to be important human pathogens that can cause a spectrum of diseases, ranging from mild skin or throat infection to more severe deep tissue infections, necrotizing fascitis, sepsis, and toxic shock—like syndromes, in addition to postinfection sequelae, such as rheumatic heart disease and poststreptococcal nephritis [1–4]. Detailed epidemiologic analysis of group A isolates at the gene and protein levels has identified differences among genes of the M protein family that are located within the *mga*-controlled regulon [5–10]. An association between expression of certain *mga*-controlled regulon gene products with IgG-binding properties and the isolates associated with skin infection has been reported [5, 6] as has evidence that certain M proteins can target attachment of specific cell types in human skin [11].

Isolate 64/14 has been extensively characterized for expression of IgG-binding proteins at both the protein and gene levels [12–14]. Three functionally, antigenically, and genetically distinct proteins can be expressed by this isolate. The *mrp* gene encodes a type Ila IgG-binding protein, which binds human IgG1, IgG2, and IgG3 in a nonimmune fashion. The immediately downstream *emm* gene also encodes a type Ila IgG-binding protein; however, this identical functional activity is mediated by an antigenically distinct protein that only demonstrates sequence similarity in the membrane anchor/cell wall–spanning region and in the signal peptide [12]. The third IgG-binding protein gene, *emm*, encodes an IgG3 only or type Ib IgG-binding protein [13]. An isogenic mutant in which the *mga* gene has been disrupted fails to express any of these IgG-binding gene products [15].

In studies of isolate 64/14, using a mouse model of skin infection, we have reported an association between expression of IgG-binding proteins and invasive potential [16, 17]. The importance of these gene products in virulence was only observed when the isolates were injected in the skin. Similar studies with the same isolates injected intraperitoneally (ip) failed to demonstrate any difference in virulence. In a parallel series of studies, the expression of IgG-binding proteins by isolate 64/14 demonstrated that organisms that were more invasive in the mouse skin infection model were more resistant to opsonophagocytosis in human blood [17]. The key gene product(s) responsible for resistance to phagocytosis were studied further using isogenic mutants of isolate 64/14 in which either the *mrp* or *emm* gene had been insertionally inactivated. Evidence for antiphagocytic roles for both the *emm* and *mrp* gene products were identified [15]. Herein, the potential role of *Mrg* 64/14 and Emm 64/14 in providing an invasive phenotype has been tested in a mouse air sac model using these well-characterized isogenic mutants of isolate 64/14.

**Materials and Methods**

**Bacteria.** Isolate 64/14 was obtained by passage of an M nontypeable clinical group A streptococcal isolate injected ip sequentially on 14 occasions as described previously [18]. Isogenic mutants of 64/14 in which the *mga*, *mrp*, or *emm* gene was insertionally activated were generated as described previously [15]. The growth rates of the wild type isolate and all isogenic mutants were similar in Todd Hewitt broth.

**Immunoglobulins.** Human IgG myeloma proteins of each subclass were obtained from the World Health Organization/International Union of Immunologic Societies Ig committee.
Proteins were radiolabeled with \(^{125}\text{I}\)iodine (Amersham, Chicago) using a lactoperoxidase method [19]. A PD-10 desalting column (Pharmacia, Piscataway, NJ) was used to separate labeled proteins from free iodine. The specific activity of all radiolabeled proteins was \(\sim 0.3\) mCi/mg.

**Mice.** Outbred CD1 mice were obtained from Charles River (Portage, Michigan).

**Air sac procedure.** The air sac procedure was a modification of a previously described method [20]. In brief, an air- and liquid-tight connective tissue pouch was generated on the back of a mouse by slow subdermal injection of 0.9 mL of air via a 27-gauge needle on a 1.0-mL syringe. The syringe containing the air also contained 0.1 mL of an appropriately diluted suspension of group A streptococci. Mice were provided with food and water ad libitum. Each experiment was continued for 96–144 h after infection, and death was used as the end point. All surviving animals were euthanatized by cervical dislocation at the end of the experiment.

The spleen was removed from any animal that died of infection and from all animals euthanatized at the end of the experiment. A spleen cell suspension was generated by homogenizing the spleen in 1 mL of sterile 10 mM PBS, pH 7.4. An aliquot was cultured to determine the purity of the culture and if bacteria were present. Mutant isolates were cultured in the presence and absence of spectinomycin to determine whether there was any evidence of revertants. The remainder of the spleen cell suspension was mixed with an equal volume of sterile glycerol and stored at \(-70^\circ\text{C}\) for later analysis.

**Analysis of IgG-binding protein expression by group A streptococci recovered from infected animals.** Bacteria were recovered from infected spleens by plating an aliquot of the glycerol-stocked spleen cell suspension on blood agar plates. In studies involving isogenic mutants of isolate 64/14, 60 \(\mu\)g/mL spectinomycin was included in the culture media. All of the group A colonies from the plate were used to inoculate Todd Hewitt broth and expanded by growth as stationary cultures overnight at 37°C.

**Polyacrylamide gel electrophoresis and Western blotting techniques.** Protein samples were denatured by boiling for 5 min in 0.5 \(M\) Tris-HCl (pH 6.8) containing 2% SDS (wt/vol), 5% \(\beta\)-mercaptoethanol (vol/vol), 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromphenol blue. Denatured proteins were electrophoresed in 10% polyacrylamide slab gels at 50 V for 16 h as described previously [21]. Prestained molecular mass standards (Bio-Rad, Richmond, CA) containing phosphorylase B \((M_r \sim 106,000)\), bovine serum albumin \((M_r \sim 80,000)\), ovalbumin \((M_r \sim 49,500)\), carbonic anhydrase \((M_r \sim 33,500)\), soybean trypsin inhibitor \((M_r \sim 27,500)\), and lysozyme \((M_r \sim 18,500)\) were included in each SDS-polyacrylamide gel assay.

The proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) by a modification of the method described by Towbin et al. [22]. In brief, SDS slab gels were presoaked for 30 min in 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3), assembled into the high field intensity transblot system (Bio-Rad), and electrophoresed in that buffer at 70 V for 3 h. The nitrocellulose blots were washed four times for 15 min each at ambient temperature with 250 mL of 50 mM veronal buffer (VBS), pH 7.4, containing 0.15 \(M\) NaCl, 0.25% gelatin, and 0.25% Tween-20 (VBS-gel-Tween) to saturate remaining protein-binding sites on the nitrocellulose. The blocked membranes were incubated for 3 h at ambient temperature in 25 mL of VBS-gel-Tween containing 3 \(\times 10^7\) cpm/mL of the appropriate \(^{125}\text{I}\)-labeled probe. The nitrocellulose membranes were then washed four times (15 min each time) with 250 mL of VBS containing 0.01 \(M\) ethylenediamine-tetraacetate (pH 7.2), 1 \(M\) NaCl, 0.25% gelatin, and 0.25% Tween-20 on a rocking platform at ambient temperature. The membranes were dried and autoradiographed using Kodak XAR-5 film and Kodak X-Omatic intensifying screens at \(-70^\circ\text{C}\) for 1–3 days.

**Isolation of DNA and Southern blot analysis.** Chromosomal DNA from group A isolate 64/14 or paired isogenic mutants in which the \(mga\) gene had been insertionally inactivated was prepared by the method of Martin et al. [23]. Plasmid DNA from *Escherichia coli* recombinants was prepared according to the method of Zhou et al. [24]. Conventional techniques for DNA manipulations, such as restriction enzyme digests, ligation, transformation of *E. coli* host cells, colony blot hybridization, 1% agarose gel electrophoresis, and Southern blotting by vacuum transfer, were performed as outlined by Ausubel et al. [25]. Transformation of group A streptococcal strains by electroporation followed the protocol of Caparon and Scott [26]. Design, synthesis, and purification of oligonucleotides were described by Podbielski et al. [27]. In that publication, the parameters for polymerase chain reaction (PCR) assays, direct labeling of PCR products with digoxigenindUTP, and analysis of PCR products were also given. Purification of PCR products was performed using the Wizard DNA purification system (Promega, Madison) according to the manufacturer’s instructions.

**Results**

The IgG-binding profile of the wild type, *emm*, or *mrp* isogenic mutants of 64/14 were compared. For these studies, bacteria were grown overnight in Todd Hewitt broth and extracted with treatment with CNBr as described in Materials and Methods. The results of this analysis are presented in figure 1 and demonstrate the expected profile of IgG-binding proteins, with no evidence for disruption of the downstream IgG3-binding *emm*...
gene product. Comparison of the expression of *emm* or *mrp* gene products in isolates in which the *emm* or *mrp* gene was inactivated demonstrated a modest increase in extractable protein encoded by the active gene. These differences were within experimental error and, consequently, were not considered to be a significant quantitative variation in gene expression by the mutants. Earlier studies of these mutants at the RNA level failed to identify any major quantitative variation in message expression for Emm in an *mrp* mutant and vice versa [15].

In the next series of studies, wild type strain 64/14 and isogenic mutants in which either the *mrp* or the *emm* gene had been insertionally inactivated were tested for virulence after 10^8 cfu were injected ip or 10^9 cfu were injected into a skin air sac (figure 2) as described in Methods. The inoculating doses were chosen on the basis of the LD_{50} determined in studies using wild type isolate 64/14 in this model [16]. The results presented in figure 2 demonstrate that inactivation of either the *emm* or the *mrp* gene had no significant effect on the virulence of isolate 64/14 when 5 × 10^8 cfu were injected ip. At a higher dose, the wild type *emm* and *mrp* mutants resulted in death for all mice on a similar time scale. These findings indicate that the inactivation strategy did not alter the growth-rate characteristics of these isolates. By contrast, injection of an isogenic mutant of isolate 64/14, in which the *emm* gene was inactivated, demonstrated significantly less virulence than the wild type organism when injected into a skin air sac. Inactivation of the *mrp* gene was found to have a significant although unexpected effect in that this mutant was significantly more virulent in the skin air sac than was the wild type organisms (figure 2B).

The results obtained in the mouse skin infection studies suggest that the effect of *emm* and *mrp* gene products on virulence in this model are opposite. If the *mrp* gene is not expressed, the organism is more virulent, while inactivation of *emm* leads to a less virulent phenotype. These results suggest that the *mrp* gene product may act to decrease the virulence of the organism, while the *emm* gene product enhances virulence. Alternatively, in vivo, the *emm* gene may be over expressed, accounting for the enhanced virulence of this mutant. To distinguish among these possibilities, we tested in the mouse skin model the virulence of an *mga* knock-out mutant in which neither the *mrp* nor the *emm* gene product was expressed.

Preliminary analysis of expression of IgG-binding proteins by the *mga* mutant confirmed that this isolate failed to express detectable quantities of the *emm*, *mrp*, or *enn* gene products (data not shown). When an *mga* mutant was injected into a skin air sac, there was no significant difference in virulence between the wild type and isogenic *mga* mutant (figure 3). This result would be consistent with the effects of the *emm* and *mrp* gene products offsetting one another and with the fact that under these circumstances, virulence mediated by other bacterial products encoded outside the *mga*-controlled regulon were being measured. Alternatively, since the *mga* mutant was generated by insertional inactivation, the results could indicate the selection of revertants displaying the wild type phenotype.

Isolates recovered from the spleens of mice that died following injection of an *mga* mutant in the skin were analyzed. Initially, the recovered mutants were tested for their ability to grow in the presence of spectinomycin by comparing colony counts on nonselective and selective media. The results of these studies demonstrated that all of the spleen-recovered *mga* mutants retained their ability to grow in spectinomycin. These
isolates were then analyzed for surface expression of IgG-binding proteins. As expected, extracts of the mga mutant used to infect the mice did not contain mrp, emm, or enn gene products (figure 4). Analysis of CNBr extracts of a number of spleen-recovered mga isolates unexpectedly demonstrated the presence of all three of the IgG-binding proteins expressed by the wild type organism (figure 4).

Enhanced expression of IgG-binding proteins has been observed when the wild type isolate was injected into the skin and recovered from the spleen of lethally infected mice [16, 17]. Since the observed IgG-binding phenotype for the mga mutant demonstrated in figure 3 was similar to that expected for skin infection of mice with the wild type parent, the possibility that reversion had occurred through a genetic rearrangement that retained the antibiotic resistance cassette was considered. Such a phenomenon has previously been reported for a kanamycin-resistance cassette inserted within the hasA gene of a group A streptococcal isolate [28].

To test for the possibility of reversion, we did Southern blot analysis to determine the location of the spectinomycin-resistance cassette on selected spleen-recovered mga isolates that demonstrate expression of IgG-binding protein. The results presented in figure 5A, using BamHI, demonstrate that the spleen recovered mga mutant containing the spectinomycin-resistance cassette and that it was genotypically unchanged from the mga mutant used to infect the mice. A similar analysis using three additional restriction enzymes (SpeI, NheI, and EcoRI) also confirmed that there was no major change at the mga gene locus following recovery from the spleen of a lethally infected mouse (data not shown).

On the basis of previous studies using isolate 64/14 [16, 17], organisms demonstrating enhanced expression of IgG-binding proteins would also be expected to demonstrate enhanced virulence when injected into the skin of naive mice [16]. This prediction was tested in the next series of experiments, and as shown in figure 6, there was a significant differ-

Figure 3. Virulence of wild type isolate 64/14 or paired isogenic mutant in which mga gene was insertionally inactivated when injected into mouse skin. Groups of 5 outbred mice were injected into skin air sac with 5 \( \times 10^6 \) cfu of wild type isolate 64/14 (■■) or with paired isogenic mutant in which mga gene was inactivated (○○). Time to death was monitored and statistical significance determined by use of Student’s t test.

Figure 4. Analysis of IgG-binding protein expression by mga mutants recovered from spleen of mice that died following inoculation of mga isogenic mutant into skin air sac. IgG-binding proteins were extracted by CNBr treatment of mga mutants recovered from spleen of mice following lethal skin infection with mga mutant. Extracts of inoculating organism are included for comparison. Results were obtained by use of human IgG1 (A) and IgG3 (B) probes. Lane 1 contains extracts from organisms recovered from spleen of 3 mice lethally infected with mga mutant analyzed in lane 1. Parallel blots probed with human IgG1 or IgG3 demonstrated pattern of reactive bands similar to that for IgG1 (data not shown).
expression of the downstream
enn
mrp
1). In the initial in vivo experiments, there was no signiﬁcant of these genes was being detected. Since the organisms would
Discussion
mice a similar pattern of infection to that of the wild type
mga
has proven to be a useful measure of expression of
mga
14 compared with that for the wild type organism or the actively facilitates clearance of the bacteria by the host, and
ence in virulence of the spleen-recovered
mga
mutant of 64/14 compared with that for the wild type organism or the
mga
mutant that had not been exposed to biologic pressures present in mouse skin.

Expression of IgG-binding proteins by group A streptococci has proven to be a useful measure of expression of
mga-controlled regulon gene products as well as being predictive of the invasive potential of group A isolates in a mouse model of skin infection [16, 17, 29]. The virulence regulon in group
A isolate 64/14 contains genes for three IgG-binding proteins. The
mrp
and the adjacent
emm
gene both encode an IgG1-,
IgG2-,
and IgG3-binding protein [12], while the
enn
gene encodes an IgG1-binding protein [13]. Expression of all three genes can be shown to be under control of the transacting
mga
gene product, and when this gene is insertionally inactivated, none of the gene products are detected [15].

In this study, we compared the virulence of wild type isolate 64/14 and paired isogenic mutants in which either the
emm
or
mrp
gene was inactivated in a mouse model. In each mutant, expression of the downstream
enn
gene was not affected, indicating that only the targeted gene had been disrupted (see ﬁgure 1). In the initial in vivo experiments, there was no signiﬁcant
difference in the virulence of the wild type and either isogenic mutant when injected ip (see ﬁgure 2A). In similar studies in which the bacteria were injected into a skin air sac, a number of interesting ﬁndings were made. First, inactivation of the
emm
gene signiﬁcantly reduced virulence of the organism compared with that for the wild type isolate (see ﬁgure 2B). By contrast, inactivation of the
mrp
gene made the mutant signiﬁcantly more virulent than the wild type organism.

Analysis of expression of Emm protein by
mrp
mutants failed to provide convincing evidence that this effect was due to overexpression of Emm by these bacteria. However, it is possible that increased expression of the
emm
gene product might be occurring due to host pressures experienced at the site of infection in the mouse and that this could account for the increased virulence observed for the
mrp
mutant.

In previous studies of IgG-binding protein expression by isolate 64/14 following sequential passage in human blood, enhanced Emm protein expression and decreased expression of the
mrp
gene products was noted, and these isolates were more virulent when injected into the skin of mice [17]. In other studies of wild type and isogenic mutants of 64/14, there was evidence that expression of either an
emm
or an
mrp
gene product can contribute to resistance to phagocytosis [15]. The difference observed between the behavior of
mrp
and
emm
mutants in human blood [15] and the expression of these gene products by the wild type organism following sequential passage in human blood [17] would be consistent with a complex regulation of
mrp
d and
emm
gen es in isolate 64/14. An alternative explanation for the ﬁndings that the
mrp
mutant was more virulent when injected into the skin of mice was that Mrp actively facilitates clearance of the bacteria by the host, and inactivation of this gene would negate this effect and make the organism more virulent.

When an
mga
mutant in which neither the
mrp
nor the
emm
gene was expressed was injected into the skin of outbred CD1 mice a similar pattern of infection to that of the wild type organism was observed (see ﬁgure 3). This result suggested that either the virulence effects of the
emm
gene and the protective effects of the
mrp
gene off-set one another or, alternatively, that the mutant had reverted to a wild type phenotype. Analysis of the bacteria recovered from the spleens of mice lethally infected with the
mga
mutant retained their ability to grow in spectinomycin. However, analysis of extracts of selected spleen-recovered isolates were found to express all three IgG-binding proteins associated with the
mga-controlled virulence regulon (see ﬁgure 4). Furthermore, the level of expression of IgG-binding proteins was increased compared with that for the isogenic wild type parent organism.

Enhanced expression of IgG-binding proteins for isolate 64/14 following passage through the skin of mice has also been reported [16]. These ﬁndings suggested that either the gene inactivation strategy was not stable and that some functional
mga
gene product was being generated or that another regulator of these genes was being detected. Since the organisms would

Figure 5. Southern blot analysis of genomic DNA from wild type 64/14 (wt) isolate, isogenic
mga
mutant, or
mga
mutant recovered from spleen of mouse following lethal infection with
mga
mutant inoculated into skin air sac. Genomic DNA (2 µg) from each isolate was digested with BamHI and subjected to agarose gel electrophoresis and Southern blot hybridizations. Probes were directed to targeted
mga
gene (right) and inserted
aad9
gene (left). 1 =
mga
mutant before mouse passage, 2 = selected
mga
mutant recovered from spleen of lethally infected mouse following injection of
mga
mutant into skin air sac. Hybridization pattern for both forms of mutant are identical and distinct from pattern of wt isolate. Only lanes 1 and 2 hybridized with
aad9
probes, indicating presence of inserted antibiotic-resistance cassette within
mga
gene.
Figure 6. Virulence of wild type (wt) 64/14, mga mutant, and mga mutant recovered from spleen of mouse following lethal skin infection with mga mutant. Groups of 5 outbred CD1 mice were injected into skin air sac with 5 × 10⁹ cfu of wt 64/14 (○), mga mutant (□), or mga mutant recovered from spleen of mouse lethally infected in skin with mga mutant (▲). Time to death was monitored and statistical significance was determined by use of Student’s t test.

The existence of an additional regulatory mechanism that can control expression of virulence factors in group A streptococci in the absence of a functional mga gene should now be considered. This may help to explain the different patterns of disease that can be caused by group A streptococci that are not reflected by major changes in serotype or genotype when the organisms are analyzed under laboratory conditions only.

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References


