Streptococcus pyogenes Infection in Mouse Skin Leads to a Time-Dependent Up-Regulation of Protein H Expression

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Streptococcus pyogenes protein H (sph) is an immunoglobulin-binding protein present in the Mga regulon of certain M1 serotype isolates. Although sph is present in many strains, it is frequently not expressed. In this paper we show that protein H was highly expressed after bacteria were injected into the skin of mice and were recovered from the blood, kidney, or spleen at various times postinfection. The percentage of protein H-positive colonies increased with time, reaching 100% in the spleen and kidney within 24 to 72 h postinfection. The up-regulation of sph expression was also observed in a mga mutant.

M1 serotypes of the group A streptococcus (Streptococcus pyogenes) have been disproportionately represented in invasive infections occurring in the past decade (17). Though most of these isolates contain a single emm gene, encoding the anti-phagocytic M protein, some isolates possess an additional M-like gene. This gene, sph, is located directly downstream of emm and encodes protein H. This protein has been found to bind Immunoglobulin G (IgG), fibronectin, and albumin (1, 5, 7) and has been implicated as a spreading factor in invasive infections (6, 9). Boyle et al. and Raeder and Boyle have also identified IgG Fc-binding proteins as important factors in the development of invasive infections in a mouse model of group A streptococcal skin infection (2, 11).

Although the expression of virulence factors in response to biological pressures in an infected host is not easy to mimic under laboratory conditions, a number of studies of S. pyogenes and isogenic mutants have documented in vivo selection of stable variants with unexpected phenotypes (2, 3, 11, 12, 15). For example, a mutant containing an insertion in the mga gene that failed to express any surface M or M-related proteins was found to overexpress these surface proteins after being recovered from the spleen of a lethally infected mouse following injection in the skin (2). This selection was achieved without any change in the position or orientation of the antibiotic resistance cassette used to inactivate the mga gene via insertion (2).

Similar studies in which a beta-hemolysis-negative pel mutant was injected into the skin of a mouse led to the recovery of a phenotype beta-hemolysis-positive variant from the kidney 72 h later (3). This change in phenotype was achieved without any change in the orientation or insertion site of the Tn917 transposon in the pel gene (3). Selection of stable SpeB-negative variants of S. pyogenes isolates has also been reported for wild-type isolates selected by injection into the skin and recovery from the spleen or following extensive passage in human blood (12).

In a recent study from our laboratory, we have shown that a clinical M1 serotype isolate, 2109, contained the sph gene but failed to express protein H when grown under normal laboratory conditions (16). However, following injection into the skin of a mouse and recovery from the spleen, stable protein H-expressing variants could be recovered (16). It was not clear from the study whether protein H was essential to establish an invasive infection or the stable expression of surface protein H resulted from exposure to unique biological pressures present in the systemic circulation of the mouse. Frick and colleagues have recently proposed a role for protein H in the dissemination of streptococci from their initial site of colonization (8), suggesting that induction of expression may be important in establishing an invasive infection.

To address this question, the skin air sacs of groups of outbred CD1 mice were injected with 10⁶ CFU of M1 serotype isolate 2109. At selected times postinfection, three mice per time point were sacrificed, and kidney, spleen, and blood samples were harvested. Following homogenization of tissue in sterile phosphate-buffered saline, each sample was diluted and a 100-μl aliquot was spread on a Todd-Hewitt agar plate and grown overnight at 37°C. Plates containing 50 to 200 well-placed colonies were tested for expression of protein H by a colony immunoblotting assay described previously (14, 18, 19).

The specific antibody probe used in these experiments was a chicken antibody monospecific for recombinant protein H, which has been shown to be specific for the targeted surface antigen (13, 16). This antibody was biotinylated, and a streptavidin enzyme reporter system was used to identify colonies expressing surface protein H as previously described (16). In a typical colony blot assay, 95 to 100% of the colonies recovered from the spleen of a mouse at 24 and 48 h after infection were positive for protein H expression (see Table 1). No reactivity was observed when a control biotinylated chicken antibody of irrelevant specificity was used (data not shown).

The colony immunoblotting procedure was used with bacteria recovered from spleen, kidney, and blood samples from mice sacrificed at different times postinfection. The pattern of expression of protein H for bacteria from each site and from
each of the three mice harvested at different times is summarized in Table 1. In agreement with previous results (16), colonies demonstrating immunologically specific reactivity with the anti-protein H antibody contained protein H in a CNBr extract of the bacteria (data not shown).

These initial kinetic studies with isolate 2109 indicated that the expression of protein H steadily increased within an hour after infection. The percentage of protein H-positive isolates increased over time, reaching 100% in the kidney and spleen after 72 h (Table 1). The percentage of protein H colonies in the blood peaked at 24 h (60%) (Table 1). The fact that only 30 to 40% of the bacteria in the blood were protein H positive at 48 and 72 h while 90 to 100% were positive in the spleen suggests that expression of protein H increased the colonization and/or survival within these organs. What is not clear from these experiments is whether protein H is essential for crossing the epithelial layer in the skin air sac. Since both protein H-positive and -negative colonies were found in the blood at 1 h, it is possible that protein H expression is not an absolute requirement for crossing a skin barrier.

Alternatively, the protein H-negative isolates recovered from the blood may have expressed protein H in the mouse and reverted to a protein H-negative phenotype when cultured in the laboratory in the absence of a biological selective pressure(s). It is also possible that protein H-negative isolates were carried into the systemic circulation with protein H-positive bacteria as part of an aggregate or chains. Since protein H has been identified as an important component of bacterial aggregation (8), such a mechanism is possible. Furthermore, tissue damage caused by a protein H-positive organism could non-specifically disrupt the barriers that normally hinder bacteria from entering the bloodstream and systemic circulation.

Furthermore, we cannot unequivocally exclude the possibility that a small subpopulation of protein H-expressing variants were present in the initial inoculum. However, if expression of protein H were an essential requirement for skin-invasive potential, or if it significantly enhanced the process, then a several-log-fold difference in the 50% lethal dose between infecting and recovered bacteria would be anticipated. In a number of previous animal studies using individual colonies from protein H gene-positive isolates that failed to express detectable protein H or isolates, no such enhancement in virulence was observed when the variants recovered following passage into the skin were injected into naive mice (11).

To further address the importance of protein H and other M and M-related proteins in establishing invasive infections, we carried out a similar series of studies with an mga knockout mutant of another M1 serotype isolate of S. pyogenes. API1. Isolate AP1 is the prototypic protein H isolate (1), and by using a Tn916 mutagenesis strategy, Kihlberg et al. (10) generated an isogenic mutant of isolate API1 in which the mga gene was inactivated (mga::Tn916). The insertion of the transposon into the mga gene leads to the complete loss of expression of both the surface M1 protein and protein H (10).

To determine if a mga mutant of isolate API1 could be invasive without expressing either surface M1 protein or surface protein H, a similar series of kinetic studies was carried out using a mga::Tn916 mutant of isolate API1. The appearance of protein H-positive colonies was monitored with the colony blotting assay described above, while the expression of M1 protein was monitored on replica blots by using an anti-M1 protein-specific antiserum as the antibody probe. In all of the studies, tetracycline (5 μg/ml) was included in the growth media and was present in the plates used for the colony blot analysis to ensure that only the variants still containing the Tn916 transposon would grow.

The results of these studies indicate that the induction of expression of both surface M1 and protein H could occur (Table 2). Colonies that expressed protein H also expressed the M1 protein. This occurred without a change in position or orientation of the transposon, as determined by PCR analysis of isolated colonies that were recovered from the spleens of lethally infected mice (Fig. 1A). Furthermore, quantitative real-time PCR analysis demonstrated that the observed expres-

**TABLE 1. Kinetics of conversion to protein H-positive phenotype following injection of isolate 2109 into a mouse skin air sac**

<table>
<thead>
<tr>
<th>Time postinfection (h)</th>
<th>No. of protein H-positive colonies/total no. (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Spleen</td>
<td>Kidney</td>
<td>Blood</td>
</tr>
<tr>
<td>1</td>
<td>21/161 (13)</td>
<td>22/48 (46)</td>
<td>8/43 (19)</td>
<td>0/0</td>
</tr>
<tr>
<td>12</td>
<td>82/149 (55)</td>
<td>316/395 (80)</td>
<td>28/61 (46)</td>
<td>0/0</td>
</tr>
<tr>
<td>24</td>
<td>6/10 (60)</td>
<td>196/199 (98)</td>
<td>283/307 (92)</td>
<td>0/0</td>
</tr>
<tr>
<td>48</td>
<td>13/33 (39)</td>
<td>384/384 (100)</td>
<td>55/59 (93)</td>
<td>0/0</td>
</tr>
<tr>
<td>72</td>
<td>4/13 (31)</td>
<td>265/265 (100)</td>
<td>224/224 (100)</td>
<td>0/0</td>
</tr>
</tbody>
</table>

* Protein H-positive colonies were found as soon as 1 h postinfection, and by 24 h postinfection, >90% of colonies recovered from the organs expressed protein H.

**TABLE 2. Kinetics of conversion to a protein H-positive phenotype following injection of the mga mutant of isolate AP1 into a mouse skin air sac**

<table>
<thead>
<tr>
<th>Time postinfection (h)</th>
<th>No. of positive colonies/total no. (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Spleen</td>
<td>Kidney</td>
<td>Blood</td>
</tr>
<tr>
<td>8</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
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</tr>
<tr>
<td>16</td>
<td>0/0</td>
<td>18/60 (33)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>24</td>
<td>0/0</td>
<td>80/100 (80)</td>
<td>12/60 (20)</td>
<td>0/0</td>
</tr>
<tr>
<td>30</td>
<td>31/97 (32)</td>
<td>500/500 (100)</td>
<td>211/270 (78)</td>
<td>31/97 (32)</td>
</tr>
<tr>
<td>48</td>
<td>22/56 (39)</td>
<td>160/160 (100)</td>
<td>255/255 (100)</td>
<td>22/56 (39)</td>
</tr>
</tbody>
</table>

* No colonies could be recovered at 8 h postinfection, and protein H-positive colonies were not found until 16 h postinfection. In each case, expression of protein H occurred concurrently with expression of M1 protein. By 48 h postinfection, all isolates recovered from the organs expressed both M1 protein and protein H. Analysis confirmed that Tn916 remained inserted in the mga gene.
Data were normalized to real-time reverse transcriptase (RT)-PCR analysis of followed by 20 min of extension at 72°C. Products were analyzed on a °C for 1 min, 52°C for 18 min; °C for 1 min, and 70°C by 35 cycles of 94°C for 5 min; followed by the addition of reverse transcriptase (no RT). RNA isolation. To control for DNA contamination, parallel experiments were run without the addition of reverse transcriptase (no RT). RNA isolation and quantitative real-time RT-PCR analysis was done as described previously (16). Primers were designed by using the mga gene of the sequenced M1 serotype isolate, SF370 (4). The primers and probe used were as follows: mga forward primer, CAAAGCAACAGTTGGAGAG; mga reverse primer, GGGTTTGTATTGGAGCA. XL-PCR mixtures contained final primer concentrations of 0.2 μM, 1.0 mM Mg acetate, an 800 μM concentration of each deoxynucleoside triphosphate, and 4 U of polymerase. Reaction conditions were as follows: 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 70°C for 18 min; followed by 20 min of extension at 72°C. Products were analyzed on a 0.8% agarose gel stained with ethidium bromide. (B). Quantitative real-time reverse transcriptase (RT)-PCR analysis of mga expression. Data were normalized to gapdH mRNA expression and are presented relative to mga expression in the wild-type AP1 strain. The Tn916 insertion into the mga gene (mga::Tn916) led to a 4- to 5-order of magnitude decrease in mga mutant mRNA compared to the wild-type mRNA level. Injection of the mga::Tn916 mutant into a mouse and recovery from the spleen (SR mga::Tn916) did not restore mga expression. To control for DNA contamination, parallel experiments were run without the addition of reverse transcriptase (no RT). RNA isolation and quantitative real-time RT-PCR analysis was done as described previously (16). Primers were designed by using the mga gene of the sequenced M1 serotype isolate, SF370 (4). The primers and probe used were as follows: mga forward primer, TGCCTTTGATAGCATCAAACAAG; mga reverse primer, CAAGGAGATGAACCACTTAA; probe, CTCACCAACGGGCTGTCGAAAAGTG. The probe was 5’ labeled with 6-carboxy-fluorescein and 3’ labeled with black hole quencher 1. Experiments were performed in quadruplicate, and the data show the average results for two separate RNA isolations.

FIG. 1. Analysis of mga expression in the wild type and mutants of S. pyogenes API. (A) The increased sizes of the PCR products from the mga::Tn916 and spleen-recovered mga::Tn916 (SR mga::Tn916) mutants relative to that of the wild type (WT) was consistent with the presence of the Tn916 transposon in the mga gene. The mga gene was amplified in the wild type and a mga::Tn916 mutant of API1 with the GeneAmp XL-PCR kit (Applied Biosystems, Foster City, Calif.). Primers were designed by using the mga gene of the sequenced M1 serotype isolate, SF370 (4). The primers used were as follows: mga forward primer, CAAATCAACAGTTGGAGAG; mga reverse primer, GGGTTTGTATTGGAGCA. XL-PCR mixtures contained final primer concentrations of 0.2 μM, 1.0 mM Mg acetate, an 800 μM concentration of each deoxynucleoside triphosphate, and 4 U of polymerase. Reaction conditions were as follows: 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 70°C for 18 min; followed by 20 min of extension at 72°C. Products were analyzed on a 0.8% agarose gel stained with ethidium bromide. (B). Quantitative real-time reverse transcriptase (RT)-PCR analysis of mga expression. Data were normalized to gapdH mRNA expression and are presented relative to mga expression in the wild-type AP1 strain. The Tn916 insertion into the mga gene (mga::Tn916) led to a 4- to 5-order of magnitude decrease in mga mutant mRNA compared to the wild-type mRNA level. Injection of the mga::Tn916 mutant into a mouse and recovery from the spleen (SR mga::Tn916) did not restore mga expression. To control for DNA contamination, parallel experiments were run without the addition of reverse transcriptase (no RT). RNA isolation and quantitative real-time RT-PCR analysis was done as described previously (16). Primers were designed by using the mga gene of the sequenced M1 serotype isolate, SF370 (4). The primers and probe used were as follows: mga forward primer, TGCCTTTGATAGCATCAAACAAG; mga reverse primer, CAAGGAGATGAACCACTTAA; probe, CTCACCAACGGGCTGTCGAAAAGTG. The probe was 5’ labeled with 6-carboxy-fluorescein and 3’ labeled with black hole quencher 1. Experiments were performed in quadruplicate, and the data show the average results for two separate RNA isolations.

sion of protein H occurred in the absence of mga expression in the same isolate (Fig. 1B).

It is of interest that no bacteria were detected in the bloodstream prior to 24 h after the API mga mutant was injected but that organisms could be detected in the kidney and spleen within 16 h (Table 2). The majority of bacteria recovered early in the infection course failed to express surface protein H or M1 protein. However, as the time of infection progressed, the frequency of protein H- and M1-expressing isolates that colonized the spleen and kidney increased to 100%.

These results suggest that the major effects of expression of M and M-related proteins is observed at the level of long-term survival and colonization of the spleen and kidney. The appearance of a large percentage of API mga mutant bacteria in the bloodstream that fail to express protein H or M1 protein suggests that these surface virulence factors may not be essential for invasion but are important for survival once the organisms disseminate and colonize organs.

It is of interest that, once selected, these IgG-binding protein variants continue to stably express high levels of surface M1 protein and protein H despite the absence of any biological selection pressure. Furthermore, the ability to select stable variants of S. pyogenes that can express key antiphagocytic proteins independent of mga regulation suggests that phenotypes that are not easily detectable under conditions of laboratory growth can occur in the infected host. These findings may provide a partial explanation for the continued emergence of virulent S. pyogenes isolates.

We thank Lars Bjorck for providing us with the mga mutant of isolate API1 and the recombinant protein H used to prepare the anti-protein H antibody. We also thank Robert Blumenthal for many helpful discussions.

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REFERENCES


