Role of Hyaluronidase in Subcutaneous Spread and Growth of Group A Streptococcus

Clarise Rivera Starr¹ and N. Cary Engleberg¹,²*¹

Departments of Microbiology and Immunology¹ and Internal Medicine,² University of Michigan Medical School, Ann Arbor, Michigan 48109

Received 28 April 2005/Returned for modification 23 July 2005/Accepted 15 August 2005

Group A streptococcus (GAS) depends on a hyaluronic acid (HA) capsule to evade phagocytosis and to interact with epithelial cells. Paradoxically, GAS also produces hyaluronidase (Hyl), an enzyme that cleaves HA. A common assumption is that Hyl digests structurally identical HA in human tissue to promote bacterial spread. We inactivated the gene encoding extracellular hyaluronidase, hylA, in a clinical Hyl⁺ isolate. Hyl⁺ and an isogenic Hyl⁻ mutant were injected subcutaneously into mice with or without high-molecular-weight dextran blue. The Hyl⁺ strain produced small lesions with dye concentrated in close proximity. The Hyl⁻ strain produced identical lesions, but the dye diffused subcutaneously. However, Hyl⁺ bacteria were not isolated from unaffected skin stained by dye diffusion. Thus, Hyl digests tissue HA and facilitates spread of large molecules but is not sufficient to cause subcutaneous diffusion of bacteria or to affect lesion size. GAS capsule expression was assayed periodically during broth culture and was reduced in Hyl⁺ strains relative to Hyl⁻ strains at the onset and the end of active capsule synthesis but not during peak synthesis in mid-exponential phase. Thus, Hyl is not sufficiently active to remove capsule during peak synthesis. To demonstrate a possible nutritional role for Hyl, GAS was shown to grow with N-acetylglucosamine but not D-glucuronic acid (both components of HA) as a sole carbon source. However, only Hyl⁺ strains could grow utilizing HA as a sole carbon source, suggesting that Hyl may permit the organism to utilize host HA or its own capsule as an energy source.

The gram-positive bacterium Streptococcus pyogenes or group A streptococcus (GAS) is a versatile human pathogen that causes a variety of diseases including cellulitis, impetigo and necrotizing fasciitis. Since the development of effective antibiotics, most GAS infections have become less lethal, but there have been epidemics of severe skin infection and toxic shock syndrome within the last 15 years that have been responsible for significant morbidity and mortality (15). Incidence of streptococcal infections in its milder forms still remains consistently high in the United States, but morbidity can be excessive in developing countries.

GAS secretes several virulence factors that facilitate infection of human tissue. In addition to a large complement of exotoxins, GAS produces a hyaluronic acid (HA) capsule that thrwarts phagocytosis by neutrophils and facilitates adherence to mucosal surfaces (25). Mutant GAS that do not have a capsule are readily phagocytosed (16, 26). Paradoxically, GAS also produces hyaluronidases (Hyl), lyse enzymes that cleave the β1,4 bond between N-acetylglucosamine and D-glucuronic acid, the repeating disaccharide that comprises the hyaluronic acid capsule. It has been determined that the GAS HA capsule is structurally identical to mammalian hyaluronic acid, which is a known substrate for streptococcal hyaluronidase (19).

Since hyaluronidases are enzymes that catalyze the breakdown of hyaluronic acid in the body, they may increase the permeability of tissue to fluids. Hyaluronidase in snake and insect venom is thought to function as a “spreading factor” by degrading host hyaluronic acid, thus allowing spread of toxin (13). Another gram-positive bacterium, Clostridium perfringens produces hyaluronidases that are also thought to contribute to the spreading of bacteria in tissues (2). Based on these observations and the tendency for GAS to spread rapidly in soft tissue, it has been widely assumed that its hyaluronidase serves a similar function (7, 14, 20, 27). Although standard texts often attribute bacterial spread in tissue to the function of this enzyme, there is no experimental data to support this assumption. If streptococcal hyaluronidase has a role in vivo, it is not clear whether it relates to its degradative effect on HA in the mammalian extracellular matrix or on HA in the bacterial capsule or on both. If hyaluronidase is an important enzyme and its primary target is mammalian HA, then the presence of this enzyme could facilitate infection. However, if the primary target of hyaluronidase is the bacterial capsule, then the secretion of active enzyme may reduce the expression of capsule, making GAS more susceptible to neutrophil phagocytosis. In the latter case, hyaluronidase might function as an “anti-virulence factor,” or, since the capsule contains large amounts of sugars at certain points in the growth cycle, hyaluronidase may serve to break down the capsule during periods of nutrient deprivation (assuming that the sugar subunits can be used as a sufficient carbon source). None of these possible roles has been investigated.

At least three hyaluronidase genes have been identified in GAS, one that encodes a secreted hyaluronidase (hylA) and two or more that are bacteriophage associated (hylP, hylP2, etc.) that are not homologous to hylA at the level of DNA sequence or protein product. Extracellular hyaluronidase activity is attributable to the product of the hylA gene, HylA, whereas HylP and its analogues are believed to be phage associated, facilitating penetration of the capsule by streptococ-
TABLE 1. Streptococcal strains and plasmids used for this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>M type</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 Hyl⁺</td>
<td>M-NTaste</td>
<td></td>
<td>Necrotizing wound infection (University of Michigan)</td>
</tr>
<tr>
<td>06.1 Hyl⁻</td>
<td>M-NT</td>
<td>Hyl⁻ derivative of strain 06 (this study)</td>
<td></td>
</tr>
<tr>
<td>2600 M1</td>
<td></td>
<td></td>
<td>Derivative of MGA166 (17)</td>
</tr>
<tr>
<td>2600.1 M1</td>
<td></td>
<td></td>
<td>Hyl⁻ derivative of strain 2600 (this study)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRS233</td>
<td></td>
<td></td>
<td>Streptococci suicide plasmid (18)</td>
</tr>
<tr>
<td>pCRS2</td>
<td></td>
<td></td>
<td>Plasmid used to inactivate hylA (this study)</td>
</tr>
</tbody>
</table>

a M-NT, M nontypeable.

T able 2. Primers for cloning and sequencing of hylA and emm genes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'HylA</td>
<td>AGTCCCTGACATGTTTTTGCCATTTCCCTGTTT</td>
</tr>
<tr>
<td>3'HylA</td>
<td>AGTCCGGATCCAAATATGCTGTTGACCCCCCTT</td>
</tr>
<tr>
<td>5'10403HylA</td>
<td>CCTGGCTGCGATATCTCCTGTTT</td>
</tr>
<tr>
<td>hylA-R5</td>
<td>CGATGTTAAAGGCAGATTGTTG</td>
</tr>
<tr>
<td>hylA-R3</td>
<td>GCCAACCAGAAATCTGTT</td>
</tr>
<tr>
<td>hylA-R2</td>
<td>GGGGCTAGTGCAGTTTCTA</td>
</tr>
<tr>
<td>hylA cent-beg</td>
<td>TAGTGGCAATTATTCACATAC</td>
</tr>
<tr>
<td>5'M1 Universal</td>
<td>GAATTTGAAATTTGCACTA</td>
</tr>
</tbody>
</table>

GAS HYALURONIDASE CHARACTERIZATION 41

MATERIALS AND METHODS

Strains. Clinical isolates of group A streptococci were selected for study because of their secretion of extracellular hyaluronidase. Strain 06 is a M-nontypeable clinical strain derived from a leg wound that produces high levels of extracellular hyaluronidase. MGA166 is a clinical M1 strain that produces no detectable extracellular hyaluronidase (Table 1). All strains were grown in Todd-Hewitt (Difco, Detroit, MI) broth supplemented with 0.2% yeast extract (THYB) or on Todd-Hewitt agar plates. For antibiotic selection, 0.1 μg of erythromycin/ml was incorporated into agar plates or broth when needed.

Escherichia coli strain DH5α was used as host for cloning vectors. pGEM-T Easy (Promega, Madison, WI) and pRS233 (18) were used as cloning and shuttle vectors, respectively. These plasmids were maintained by growing E. coli in Luria-Bertani media with ampicillin (0.1 mg/ml) or erythromycin (0.3 mg/ml) as appropriate.

Inactivation of hyaluronidase gene. Strains 06.1 and 2600.1 (hylA→pCRS2) were generated from strains 06 and 2600, respectively, by insertional mutagenesis. A 1.5-kb internal fragment of hylA gene was amplified by PCR using primers 5'HylAint and 3'HylAint which have BamHI and PstI sites at the 5' ends (Table 2).

The amplicon was purified by using a Microcon filter with a 100-kDa cutoff (Amicon, Inc., Beverly, MA) and was ligated into BamHI- and PstI-digested pGEM-T Easy Vector overnight at 16°C to generate pCRS2. The plasmid was isolated from a colony containing the inserted fragment, and the BamHI and PstI fragment was excised and ligated into pRS233, a temperature-sensitive plasmid that confers resistance to erythromycin. The resulting construct, pCRS2, was electroporated by using previously described methods (5) into clinical strain 06 and 2600. Colonies were screened for growth at the nonpermissive temperature, and coinoculates were screened for hylA interruption by PCR and confirmed by using a hyaluronidase agar diffusion/activity assay when appropriate (9). A Hyl⁻ strain containing the insertion in hylA originating from Hyl⁺ 06 strain was designated strain 06.1 and maintained on media containing erythromycin.

determine whether there were any phenotypic effects other than hyaluronidase interruption caused by integration of plasmid into hylA and to control for the presence of the plasmid on the chromosome, plasmid pCRS2 was also electroporated into Hyl⁻ strain 2600, and integration of plasmid was designated strain 2600.1 and maintained on medium containing erythromycin. To isolate a revertant, these strains were passed on THYA plates without erythromycin several times until antibiotic-sensitive colonies were detected by replica plating. These isolates were presumably derived by spontaneous excision and curing of the plasmid, since most had restoration of hyaluronidase activity by the diffusion/activity assay and/or PCR analysis showed loss of the integrated plasmid.

Assays for hyaluronidase activity. Hyaluronidase activity was determined by using an agar diffusion/activity assay (9). Briefly, 1% Noble agar solution was combined with 0.4 mg of human umbilical cord hyaluronic acid/ml, Na⁺ salt (Sigma, St. Louis, MO), 1% (vol/vol) bovine serum albumin, and 0.1% sodium azide. Standard petri dishes were poured and cooled, and 6-mm wells were cut into the agar. For the activity assay, 0.1 ml of overnight culture supernatants was added to the wells, followed by incubation at 37°C for 17 to 18 h. The plate was then flooded with a 2 N acetic acid solution for 30 min. Undigested hyaluronidase forms a precipitate under these conditions, creating hazy opacity in the agar and leaving a zone of clearance around wells containing hyaluronidase. We used 0.1 ml of a 1-mg/ml solution of bovine testicular hyaluronidase as a control. Plates used to screen individual colonies for hyaluronidase activity were prepared as described above with the addition of Todd-Hewitt media with 1% yeast extract without sodium azide (THY-HA agar). Bacteria were spotted onto THY-HA and grown overnight. Before the addition of 2 N acetic acid to the THY-HA plate, colonies were transferred in situ to a THY agar plate using nitrocellulose membrane.

To quantify bacterial cell-associated HA, the Stains-All assay was used (16). A total of 10 ml of culture harvested at various stages of growth was centrifuged and washed once with sterile double-distilled H₂O (ddH₂O) to remove THYB. The pellet was then resuspended in 1 ml of ddH₂O and 0.5 ml of CHCl₃ and vortexed and incubated at 37°C for 10 to 15 min. The plate was then flooded with a 2 N acetic acid solution for 30 min. Undigested hyaluronidase forms a precipitate under these conditions, creating hazy opacity in the agar and leaving a zone of clearance around wells containing hyaluronidase. We used 0.1 ml of a 1-mg/ml solution of bovine testicular hyaluronidase as a control. Plates used to screen individual colonies for hyaluronidase activity were prepared as described above with the addition of Todd-Hewitt media with 1% yeast extract without sodium azide (THY-HA agar). Bacteria were spotted onto THY-HA and grown overnight. Before the addition of 2 N acetic acid to the THY-HA plate, colonies were transferred in situ to a THY agar plate using nitrocellulose membrane.

Mouse infection model. For mouse inoculation, GAS were grown to an optical density at 600 nm (OD₆₀₀) of 0.7 (mid-log phase; 10⁹ CFU/ml), and 0.1 ml was injected subcutaneously into the right flanks of 4-week-old male hairless SKH1(br hr)Br mice (Charles River Laboratories, Wilmington, MA). Inoculated animals were weighed and observed daily for signs of skin infection or ulceration. In some experiments, dextran blue at 5 mg/ml was added to the bacteria, followed by vortexing and injection into the mouse flank. As a negative control for this experiment, dextran blue combined with unincoculated broth was injected. As a positive control, 1 mg of bovine testicular hyaluronidase (Sigma, St. Louis, MO) in THYB was combined with 5 mg of dextran blue/ml and injected into...
the flanks of hairless mice. At 48 h after inoculation, 2-mm punch biopsies were taken from center of site of injection, from the edge of visible dextran spread, and from a point midway between both biopsy sites. Tissues were then streaked onto THYA to determine presence or absence of bacteria. Bacterial cell number was measured by staining with trypan blue and counting viable colonies on agar plates.

**Sequencing of hylA and emm genes.** Total DNA was harvested from overnight cultures of GAS strains by modification of a previously described method (21). Briefly, the bacterial pellet was washed once in 0.1 ml of streptococcal lysis buffer (30% sucrose, 50 mM Na+ phosphate buffer [pH 6.0], and 1 mM MgCl2) and then resuspended in 0.1 ml of streptococcal lysis buffer with 0.01 ml of mutanolysin (1 U/ml Sigma), followed by incubation for 30 min at 37°C. Next, 0.250 ml of Triton-EDTA, 0.015 ml of 10% sodium dodecyl sulfate, and 0.003 ml of proteinase K (20 mg/ml) was added to the cell slurry, followed by incubation at 65°C for 30 min. Then, 0.050 ml of 5 M NaCl and 0.040 10% CTAB (cetyltrimethylammonium bromide) in 0.7 M NaCl was added, followed by incubation for another 10 min at 65°C. A total of 0.750 ml of phenol-chloroform-isomyl alcohol (25:24:1) PCIAA was combined with cell extract and centrifuged at 14,000 × g, and the aqueous layer was combined with ethanol to precipitate the DNA. DNA pellets were washed with 70% ethanol, dried, and resuspended in 0.1 ml of H2O. RNase H was added to remove RNA (0.001 ml of 20 mg/ml). Finally, 2 μl of DNA was used for PCR.

Primers used to amplify the hylA gene (5′10403HylA and 3′10403HylA), as well as those used for DNA sequencing of hylA and emm, are listed in Table 2. PCR products were sequenced at the University of Michigan DNA Sequencing Core. The emm and hylA gene sequences were determined by using NCBI nucleotide BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). The M type was assigned based on homology with existing emm sequences of known type. M type was classified as nontypeable if a BLAST search resulted in closest homology to “emm-like” genes rather than to any known emm sequences.

**Growth in minimal medium.** Minimal medium was prepared as described by van de Rijn and Kessler (23). Three separate salts, amino acids, and vitamin solutions were made and combined to comprise the minimal medium. Salts were combined in a 1:1 solution. Amino acids were made from a 10% solution of Casamino Acids supplemented with l-glutamine, hydroxy-l-proline, and tryptophan and diluted to a final concentration of 1% (vol/vol). Vitamins were prepared in a 50% solution and diluted to a final concentration of 1×. The growth-limiting nutrients added to different formulations of minimal media were as follows: glucose at 10 mg/ml, HA at 0.4 mg/ml, N-acetylglucosamine at 0.4 mg/ml, and d-glucuronic acid at 0.4 mg/ml (Sigma). All stock solutions were filtered through a 0.22-μm-pore-size filter before use. A total of 0.2 ml of minimal medium with or without a carbon source and 0.005 ml of washed overnight Hyl+ or Hyl- culture was added in quadruplicate wells in a 96-well microtiter plate. Plates were incubated in a SpectraMax 250 spectrophotometer at 37°C overnight and read at OD600 at 30-min intervals to establish the growth curve.

**RESULTS**

**Analysis of clinical strains collected from 1999 to 2003.** Published reports have indicated that a large majority of clinical strains do not produce an active hyaluronidase enzyme in vitro. In order to determine prevalence of hyaluronidase activity, clinical strains from the University of Michigan Hospitals from 1999 to 2003 were collected and tested. None of these strains was associated with definable outbreaks, and they were derived from a diverse group of patients varying by age and place of residence. Of the 110 tested, only 10 produced detectable levels of hyaluronidase activity (9.1%). This percentage is considerably lower than published reports (11) that stated that 25% of clinical strains produce extracellular hyaluronidase (Table 3).

The emm genes of these seven isolates were amplified by using PCR and sequenced to determine whether certain M types correlated with hyaluronidase positivity. The emm genes from 8 Hyl+ strains and 20 Hyl- strains were sequenced, and the distribution of the M types is shown in Table 3. Seven of eight Hyl+ strains sequenced were M typeable, whereas one was M49. These seven strains were deemed “nontypeable” because three had closest homology to emm50 (an emm-like gene) and the remaining four had closest homology to msp22, sir22, or other emm genes. Of the 20 Hyl- strains sequenced, half were type M1 or M3, and the other half were a range of other M types (Table 3). In this small sample, detectable hyaluronidase activity correlated strongly with nontypeable M protein (P = 0.0007, Fisher exact test).

The hylA gene of 8 Hyl+ and 20 Hyl- strains was amplified by PCR and sequenced. A single-base-pair substitution in the codon for amino acid 806 was noted in 16 of the 20 Hyl- strains sequenced. This substitution changed a glutamic acid codon (CAA) to a stop codon (TAA), resulting in a 63-amino-acid truncation. Of four M3 strains sequenced, a 70-amino-acid in-frame deletion was seen at the 3′ end of the hylA gene. Although some minor substitutions were seen between Hyl+ and Hyl- alleles upstream of these 3′ end mutations, the stop codon substitution was the most consistent and prevalent in all Hyl- strains.

**Inactivation of hylA gene.** To examine the effect of hylA on virulence, we constructed a strain with an insertion allele of hylA. The GAS hylA gene is 2.6 kb in length with variable sequence at the 3′ end of the gene. To inactivate the hylA gene, an internal, conserved fragment of the gene was ligated with the temperature-sensitive replicon pJRS233 to yield pCRS2. This plasmid was then electroporated by previously described methods (3) into Hyl+ strain (strain 06) and Hyl- strain (strain 2600). Colonies were screened for erythromycin resistance at nonpermissive temperatures (37°C), and insertion of the plasmid into the chromosome was confirmed by PCR (Fig. 1). Isolates with pCRS2 insertions that originally derived from Hyl+ strain 06 were found to have lost hyaluronidase activity when screened by plate diffusion assay. The hylA gene is flanked by the accO gene, part of the acetoin dehydrogenase complex, and Spy1033, a putative lipoate-protein ligase (Fig. 1A). Both flanking genes and neighboring genes are transcribed in the opposite direction of hylA transcription. Thus, downstream polar effects of plasmid insertion should not occur.

There were no apparent changes in colony morphology due to the presence of chromosomal insertion of pCRS2, however there was an extended lag time of growth (average of 2.5 h rather than 2 h) in all strains that contained the plasmid. We attribute this to the presence of erythromycin needed to maintain the plasmid during growth in vitro. Multiple passes of mutant strain without erythromycin resulted in curing of the plasmid (Fig. 1) and consequent reversion to an erythromycin-sensitive, hyaluronidase-producing phenotype with restored wild-type growth rate.

**Presence of hyaluronidase affects cell-associated capsule concentrations in vitro.** To determine whether hyaluronidase affects the integrity of the GAS capsule, cell-associated capsule

---

**TABLE 3. Hyaluronidase activity of clinical strains collected from the University of Michigan Hospitals and associated M types**

<table>
<thead>
<tr>
<th>Strain phenotype</th>
<th>No. of isolates (% of total)</th>
<th>No. of isolates sequenced</th>
<th>M type(s) (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HylA+</td>
<td>10 (9.1)</td>
<td>8</td>
<td>M-NT (7), M49 (1)</td>
</tr>
<tr>
<td>HylA</td>
<td>100 (90.9)</td>
<td>20</td>
<td>M1 (6), M3 (4), M-NT (3), M18 (2), M5 (2), M28 (1), M11 (1), M110 (1)</td>
</tr>
</tbody>
</table>

* M-NT, M nontypeable.
concentrations were determined at various stages of growth. Strain 06 (Hyl/H11001) and its isogenic Hyl/H11002 mutant 067.1 were grown to stationary phase, and 10-ml aliquots in quadruplicate were taken at intervals from 4 to 24 h (mid-exponential phase through stationary phase) to determine the level of cell associated HA. Previous studies have shown that GAS capsule is expressed during mid-exponential phase and that transcription ceases during stationary phase (4).

We observed significant differences in cell-associated uronic acid concentrations between strain 06 (Hyl/H11001) and its isogenic Hyl/H11002 mutant, 067.1, during mid-exponential phase (OD 600/H11005 0.5) and at the beginning of stationary phase (OD 600/H11005 1.0) (Fig. 2). In mid-late exponential phase, when capsule expression is most active, the difference between strains was not significant. Capsule expression was also measured in stationary phase at 9, 12, 16, and 24 h, after capsule synthesis gene expression has ceased. We observed a significant difference in capsule expression between Hyl and Hyl strain early in stationary phase but not at later time points. Both strains had shed their capsule to equivalent degree by 16 and 24 h. These findings indicate that hyaluronidase can degrade its own capsule but that the presence of hyaluronidase does not affect the ability of GAS to eliminate capsule in stationary phase.

**Effect of HylA on virulence in a murine model of skin infection.** It is widely assumed that the presence of hyaluronidase causes bacteria to spread more rapidly in skin infection, thus causing an increase in severity of disease and/or mortality. To determine whether inactivation of the hylA gene affects virulence, we utilized a murine model of skin infection. Strain 06 (Hyl/H11001) and isogenic Hyl/H11002 mutant 067.1 were grown to mid-exponential phase (OD600 of 0.7 or 10^8 CFU/ml), and 10^7 CFU were injected into the flanks of hairless Skh1(hr hr)Br mice to determine the severity of disease. Mice were weighed and monitored for several days. At 48 h, all mice survived injections and were healthy based on appearance and weight gain. Groups of mice receiving Hyl or mutant Hyl strains produced a small red, nodular lesion at site of injection with either strain. By 7 days, the mice in both groups had cleared the infection and gained weight, indicating that both strains produced a transient skin infection of similar size and severity.

![FIG. 1. Inactivation of hylA. (A) hylA and surrounding genes of the GAS chromosome; acoABCL-acetoin dehydrogenase complex, Spy1033 (putative lipoate-protein ligase); and Spy1035 (putative UDP-N-acetylmuramyl tripeptide synthetase). The expanded view of hylA shows the integration of pCRS2 with sites of PCR primer binding. Primers 1 and 2 (5’HylAint and 3’HylAint, respectively) were used to generate the 1.5-kb internal hylA fragment on pCRS2. This sequence in present in all strains and is duplicated when pCRS2 cointegrates. Primers 3 and 4 (5’10403HylA and M13FORUniversal) amplified a 3.0-kb product only when pCRS2 integrated into the chromosome. (B) PCR and 1% agarose gel electrophoretic analysis of DNA from strains 06, 067.1, 067.1rev, 2600, 26007.1, and 26007.1rev. The presence of the 3.0-kb fragment in strains 067.1 and 26007.1 confirms the insertion of pCRS2 into hylA in these strains.](image-url)
Because the lesion produced by the wild-type strain was relatively small, we repeated the experiment adding manipulations known to increase lesion size in this model. First, Hyl+/H11001 and isogenic Hyl-/H11002 strains were inoculated in combination with Cytodex beads (10^7), a manipulation previously shown to enhance lesion size in mice (1). Although the lesion sizes increased slightly (red nodular lesion became small 2-mm open lesion, n = 5 per group), the lesion size was the same in mice infected with both Hyl+/H11001 and Hyl-/H11002 strains. Second, we pretreated mice intraperitoneally with 0.020 mg of human plasminogen (provided by William Fay)/ml and subsequently inoculated them in the right flanks with 10^7 Hyl+/H11001 or Hyl-/H11002 bacteria (n = five animals per group). We have shown that mice injected with human plasminogen produce more severe lesions in our murine model because of the specificity of streptokinase for the human form of this protein (12). At 48 h, the lesion sizes in both groups averaged 2 mm (comparable to those obtained by using Cytodex beads), and mice appeared healthy and experienced weight gain (data not shown).

**Effect of hyaluronidase on diffusion of large molecules through murine skin.** To determine whether streptococcal hyaluronidase acts on hyaluronic acid in the subcutaneous tissues, we used high-molecular-weight dye, dextran blue, to assess diffusion through murine tissue. Dextran blue is visible when injected under the skin of hairless mice and remains localized long enough to assess variations in its spread. The supernatants of mid-exponential-grown Hyl+/H11001 strain (06), isogenic Hyl-/H11002 strain (067.1), and revertant Hyl+/H11001 strain (067.1R) were each combined with 5 mg of blue dextran/ml by vortexing. A total of 0.1 ml of the supernatants was injected into the flanks of hairless Skh1(hr hr)Br mice. As a positive control, 1 mg of bovine testicular hyaluronidase (Sigma)/ml dissolved in THYB combined with dextran blue was used. As a negative control, THYB alone combined with dextran blue was also injected. At 48 h, the mice were scored for the spread of blue dye. The supernatants of strains 06 and 067.1R, which contain hyaluronidase, caused diffusion of dextran blue from the site of injection to the backs and abdomens of the mice in a manner similar to the positive control (Fig. 3). Mice injected with supernatants from the Hyl+/H11001 strain (067.1R) retained concentrated dextran blue close to the site of injection, as did mice injected with a combination of dextran blue and THYB. The Hyl-/M1 strain 2600 and its isogenic Hyl mutant, 26007.1, supernatants were also combined with dextran blue and injected into mice and the dye remained close to site of injection as in the Hyl-/mutant 067.1 (data not shown). We conclude that GAS hyaluronidase is capable of facilitating the spread of high-molecular-weight molecules and is therefore likely acting directly on the tissue matrix.

**Diffusion of bacteria through the skin in the presence of HylA.** To test the assumption that hyaluronidase facilitates bacterial spread in tissue, Hyl+ and Hyl- GAS were grown to mid-exponential phase (OD_600 of 0.7 or 10^8 CFU/ml), and the bacterial inoculum was combined with blue dextran (5 mg/ml). A total of 10^7 CFU was then injected into the flanks of hairless Skh1(hr hr)Br mice. At 48 h, mice were sacrificed, and blue dye spread was measured on transparent media. Punch biopsies (2 mm) were then performed at the point of injection, at 10 mm, at 20 mm, and on opposite flank to determine presence of GAS (Fig. 4).

Since strain 067.1 (Hyl-) was created by insertional inactivation with plasmid pCRS2 and requires erythromycin for maintenance through multiple passages on nonselective media, we wanted to confirm that spontaneous curing did not occur in vivo. We therefore cultured punch biopsies from these wounds on THYA-Erm and THYA plates in parallel. We found no differences in colony counts, indicating that strain 067.1 retained the plasmid throughout murine infection in the absence of erythromycin selection.

All mice survived injections and were healthy based on appearance and weight gain. As in previous experiments, mice produced similar small, red, nodular lesions when injected with Hyl+ or mutant Hyl- strain. However, mice injected with Hyl-
(06) had a greater diffusion of blue dextran than those injected with isogenic Hyl\textsuperscript{−} strain 067.1 (20 mm versus 10.2 mm). Punch biopsies revealed that bacteria were confined to the center of the lesion in both groups of mice and did not extend into the area of dye spread. This indicates that the presence of hyaluronidase was not sufficient for the movement of bacteria through the skin but only for spread the high-molecular-weight dye, blue dextran.

Use of hyaluronic acid by GAS as a carbon source. Since our results suggested that hyaluronidase is not sufficient for the spread of bacteria through the skin of hairless mice but is capable of degrading capsule when bacteria are grown in vitro, we explored a potential alternate role for this enzyme. We were motivated by the observation that \textit{hylA} is located between genes responsible for carbohydrate metabolism and transport in the GAS genome (Fig. 1A). We hypothesized that hyaluron-
idase may participate in the hydrolysis of hyaluronic acid, and possibly other complex carbohydrates, for nutritional purposes. In order to test whether Hyl\(^{-}\)/H11001 strains could break down HA and use it as a carbon source, we examined the growth of GAS with or without hyaluronidase activity in minimal medium supplemented with carbohydrates or sugars as the sole carbon source.

The Hyl\(^{-}\)/H11001 strain 06 and its Hyl\(^{-}\)/H11002 derivative, 067.1, each grew in minimal medium with 10 mg of glucose/ml; however, only the Hyl\(^{-}\)/H11001 strain was able to grow in minimal medium supplemented with 0.4 mg of hyaluronic acid/ml as the only carbon source (Fig. 5). Since hyaluronic acid is comprised of the sugars N-acetylglucosamine and D-glucuronic acid, these two sugars were tested in combination and separately as carbon sources in minimal medium culture. Both the Hyl\(^{-}\) and the Hyl\(^{+}\) strains were capable of growing in N-acetylglucosamine alone, but neither grew in D-glucuronic acid alone. Hyl\(^{+}\) strains were also unable to grow in 0.4 mg of chitotriose/ml, which is a \(1,4\)-linked polymer of N-acetylglucosamines, or in 0.4 mg of pneumococcal type III capsule/ml, which is composed of alternating glucose and glucuronic acid residues (data not shown). Streptococcal hyaluronidase was unable to degrade other components of the extracellular matrix (ECM), chondroitin sulfate and heparin sulfate (data not shown). Collectively, these experiments indicate that streptococcal hyaluronidase is specific to the \(1,4\) linkage between N-acetylglucosamine and D-glucuronic acid but not to other combinations of these sugars. These data also indicates that, in order for HA to be used as a carbon source, it must first be cleaved, releasing N-acetylglucosamine, which can then be used by bacteria irrespective of hyaluronidase production (22).

![FIG. 5. Growth of strains 06 (Hyl\(^{+}\)) and 067.1 (Hyl\(^{-}\)) in minimal medium with various carbon sources. Both strains were capable of growing in glucose, a combination of the sugars N-acetylglucosamine and D-glucuronic acid and N-acetylglucosamine alone. Only Hyl\(^{+}\) strain was capable of growing in HA alone. Neither strain could grow in D-glucuronic acid alone.]

**DISCUSSION**

By analogy with what is observed in other pathogens, hyaluronidase in GAS has been assumed to facilitate the spread of the organism through the tissues of the infected host. Although this would seem to be an important function for GAS, bacteria noted for producing rapidly spreading skin lesions. However, this assumption is challenged by the small numbers of strains that produce detectable levels of this enzyme in vitro. Similarly, the apparent contradiction of producing an enzyme that could render GAS more susceptible to phagocytosis by deconstructing capsule is not explained. The purpose of the present study was to examine the assumptions about the role of hyaluronidase in streptococcal pathogenesis and to attempt to demonstrate a function for this enzyme.

We found that frequency of hyaluronidase expression among unrelated clinical strains collected from the University of Michigan Hospitals was significantly lower than published results (8). We also observed that the most clinically significantly M types (M1 and M3) do not produce an active enzyme in vitro. In these isolates and several other Hyl\(^{-}\) strains, we found a common allelic variant in 16 unrelated strains, a variation in the sequence encoding codon 806, resulting in a substitution of a glutamine codon with a stop codon. In addition, four M3 strains were found to have a 70-amino-acid in-frame deletion in the same region. It appears that most virulent streptococci have evolved with a nonfunctional hyaluronidase gene. Therefore, determining the role of this enzyme in the small minority of streptococci that maintain it is important to understanding not only the relationship of capsular HA and Hyl, but Hyl function, if any, in virulence. One hypothesis is that hyaluron-
idase provides an added benefit for the bacteria that produce it, one more virulence factor to aid in its survival either within or outside of the host. Another hypothesis is that the hylA gene may serve as an “anti-virulence factor,” causing the HA synthetic machinery to work harder to avoid losing capsule; this may explain why so many GAS strains have evolved with a truncated protein resulting in an inactive enzyme.

Transcription of the GAS capsular synthesis genes (hasAB) is turned on during mid-exponential phase and attenuated during stationary phase (4). The most abundant cell-associated capsule concentrations occur at the end of exponential phase; thereafter, capsule is shed. We considered the possibility that hyaluronidase mediates this stationary phase capsule release. We found that this was not the case by comparing cell-associated HA in wild-type and Hyl− mutant strains. If there is any significant effect of hyaluronidase on capsule degradation, we would expect to see it during exponential phase when capsule is being synthesized. We found a significant difference between Hyl+ and Hyl− strains in cell-associated capsule concentrations at mid-exponential phase (OD600 = 0.5) and at early stationary phase (OD600 = 1.0 or 9 and 12 h of incubation). However, when cultures reached an OD600 of 0.7, the effect of hyaluronidase on total cell-associated capsule was insignificant. A broth culture grown to an OD600 of 0.7 has active hasAB transcription and abundant capsule expression. Primer extension data indicates that the levels of transcription of hylA and hasAB gene during mid-exponential phase (OD600 = 0.6) were similar in Hyl+ and isogenic Hyl− strains (data not shown), indicating that transcription of capsule synthesis genes does not increase to compensate for presence of hyaluronidase. The observations in Fig. 2 suggest that streptococcal hyaluronidase degrades the streptococcal capsule, but this effect is apparent experimentally at the onset of exponential phase and at the termination of stationary phase when capsule synthesis levels are low. In mid-to-late exponential phase, when capsule expression is maximal, the effect of hyaluronidase is less apparent.

Inactivation of the hylA gene in strain 067.1 resulted in no measured difference in lesion size or morbidity in mice infected with wild-type or mutant. Mice infected with either Hyl+ or Hyl− strains produced red, nodular lesions that were cleared after 2 weeks. Attempts to enhance the virulence either with Cytodex beads or human plasminogen produced a more virulent infection, but the same enhancement occurred in mice inoculated with either Hyl+ or Hyl− strains. This indicated that the presence of hyaluronidase did not affect virulence altered with these conditions in our mouse model.

Initial punch biopsies of murine skin infected with dextran blue and Hyl+ or Hyl− strains were performed at a predetermined distance from the site of inoculation, covering all areas of dextran blue diffusion, and beyond, in either a posterior or an anterior direction. In addition, punch biopsies in subsequent experiments were performed measuring in 10-mm increments from the site of injection, in the direction of the dextran blue diffusion. Using either method of sampling, bacteria were never found more than 10 mm from site of injection, although the dextran blue diffused twice this distance in Hyl+ strains. At the site of injection, there was visible purulence, indicating that mice mounted an acute inflammatory response that may have been capable of arresting the spread of the bacteria through the tissue. We do not know whether a comparable response would occur in humans infected with these strains. The strains used in the present study were derived from invasive streptococcal disease; however, the inability of the bacteria to spread in our mouse model may be due to factors other than lack of hyaluronidase. These findings in combination with the lack of enzyme activity in the majority of clinical strains lead us to conclude that the presence of hyaluronidase is not sufficient for dermal spread of bacteria.

The diffusion of the dextran blue dye through the skin of mice indicates that GAS hyaluronidase is capable of breaking down the HA in the ECM, allowing the diffusion of macromolecules through tissues. Since streptococcal hyaluronidase is specific for HA and not for the other carbohydrates that comprise the ECM (i.e., chondroitin sulfate and heparin sulfate), we considered the possibility that the main function of hyaluronidase is in the specific breakdown of HA in tissue for nutritional purposes. Therefore, we examined the possibility that HA could be used as a carbon source in vitro. We found that Hyl+ strains are capable of growing in minimal medium supplemented with HA. This suggests the possibility that streptococci may utilize its tissue breakdown products or even its capsule as a potential energy source. The latter capacity to consume its own capsule for energy may be important in transmission and viability of the organism outside of the human host. It is thought that spread of infection may occur via fomites, such as a toy or door handles, environments where nutrients and/or moisture may be limited. The HA capsule can protect the organism from desiccation regardless of hyaluronidase activity for a limited time (unpublished observation), but the ability of Hyl+ strains to use HA in nutrient-starved conditions may be one advantage GAS have if they produce active enzyme.

ACKNOWLEDGMENTS
We thank Victor DiRita and Phillip C. Hanna (University of Michigan) for critical reading of the manuscript. We also thank Wayne L. Hynes (Old Dominion University, Virginia) for useful discussions. C.R.S. was supported by a Rackham Merit Fellowship Grant and by an NIH Research Training Grant. N.C.E. was supported by PHS grant ROI AI14682-01.

REFERENCES


