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M Proteins of Group C Streptococci Isolated from Patients with Acute Pharyngitis

ALAN L. BISNO,[1,2]* CARLEEN M. COLLINS,[3] AND JAMES C. TURNER[4]

Medical Service, Miami Veterans Affairs Medical Center,[1] and Departments of Medicine[2] and Microbiology,[3] University of Miami School of Medicine, Miami, Florida, and Department of Student Health, University of Virginia, Charlottesville, Virginia[4]

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We studied 15 strains of group C streptococci (*Streptococcus equisimilis*) isolated from the throats of college students with acute pharyngitis and 5 strains isolated from patients with noninfectious problems. Nineteen of the 20 strains resisted phagocytic killing during incubation in normal human blood, suggesting that they might express M proteins. Genomic DNA from all 20 strains hybridized with a probe corresponding to the carboxy-terminal one-third of the group A M-protein gene *emm24*, a region that is highly conserved among M proteins of group A and group G streptococci. The DNA sequences of the N-terminal (variable) regions of the M-protein-encoding genes from two disease-associated group C isolates and one control isolate were determined. The predicted amino acid sequences of the two pharyngitis strains were identical and were 88% homologous to the amino acid sequence of a group G M-protein gene. The predicted terminal amino acid sequence of the control strain does not correspond to any such sequences in the GenBank database. All three strains studied possess the conserved region domain common to class I group A M-protein types epidemiologically associated with rheumatic fever. These studies demonstrate the presence of M proteins in strains of *S. equisimilis* isolated in cases of endemically occurring acute pharyngitis. Certain of these proteins are similar to those of group G streptococci, while others may represent new M types. The similarity in structure and function between M proteins of nonrheumatogenic serogroups and those of rheumatogenic group A streptococci suggests that factors other than or in addition to M protein per se are likely involved in the pathogenesis of rheumatic fever.

Group C streptococci are distinguished by the presence of a cell wall polysaccharide composed of *N*-acetyl-galactosamine and rhamnose, but they are heterogeneous in regard to biochemical reactions, hemolytic reactions, and predilection for host species. The large-colony forms can be differentiated into two species: *Streptococcus dysgalactiae* (alpha-hemolytic) and *Streptococcus equi* (beta-hemolytic). *Bergey's Manual of Determinative Bacteriology* (26) recognizes three subspecies of *S. equi*: *equi*, *equisimilis*, and *zooepidemicus*. Classification of the small-colony forms of group C streptococci is more complex. They are variously designated as members of the *Streptococcus milleri* group (48, 49), as members of the *Streptococcus intermedius* group (44), or as *Streptococcus anginosus* (18, 36, 41). Moreover, such small-colony strains may belong to Lancefield group A, C, F, or G or may be nongroupable.

Group C streptococci isolated from human infections may be either large colony, beta-hemolytic forms (usually *S. equi* subsp. *equisimilis*) or *S. anginosus*. The latter species has been frequently associated with bacteremia and abscess formation (36, 41). *S. equi* subsp. *zooepidemicus* is an important animal pathogen, but human infections due to this organism may occur following ingestion of unpasteurized milk or dairy products (1, 19, 20) or contact with pigs (50). Human infections due to *S. equi* subsp. *equi* (the causative agent of strangles in horses) and *S. dysgalactiae* are extremely rare.

S. equi subsp. *equisimilis* and *S. anginosus* are the only two group C streptococci regularly isolated from human sources. Both are frequently recovered from throat swab cultures of patients with pharyngitis as well as healthy controls (22, 36). The role of these bacteria in causing endemic pharyngitis is not well established (15, 25), but subspecies *equisimilis* has been implicated in purulent pharyngitis whereas *S. anginosus* has been postulated to represent normal oropharyngeal flora (46). Identification among subspecies *equisimilis* strains of molecular, biochemical, or physiologic characteristics known to be associated with virulence in recognized oropharyngeal pathogens would suggest that group C streptococci may indeed possess the potential to cause acute pharyngitis in humans.

M protein is the major virulence determinant of group A streptococci. This fibrillar, surface-exposed molecule deters opsonization of the organism by the alternate complement pathway (7, 12), and strains richly endowed with the protein are highly resistant to phagocytosis by human polymorphonuclear leukocytes, multiply readily in fresh human blood, and are capable of initiating disease in humans and experimental animals. Strains that fail to express M protein are avirulent. In the present study we show that strains of *Streptococcus equisimilis* isolated from the throats of patients with acute pharyngitis and healthy controls resist phagocytosis in human blood and possess genomic DNA encoding the carboxy-terminal portion of the M-protein molecule known to be highly conserved among M proteins of groups A and G. The predicted M-protein N-terminal sequences of three group C strains have been determined. This portion of the molecule, which is highly variable from strain to strain, determines type specificity. In one case the N-terminal sequence differs from those of all previously identified M types of group A and group G streptococci.

* Corresponding author. Mailing address: Miami VA Medical Center (111), 1201 N.W. 16th St., Miami, FL 33125. Phone: (305) 324-3264. Fax: (305) 324-3147. Electronic mail address: ABISNO @MEDNET.MED.MIAMI.EDU.

MATERIALS AND METHODS

Bacterial strains. The 20 *S. equisimilis* strains chosen for study were obtained from throat swab cultures from patients presenting to the Thomson Student Health Center at the University of South Carolina. The swabs were inoculated on sheep blood agar plates and incubated in 5 to 7% CO_2. Beta-hemolytic colonies were serogrouped by using a commercial immunoagglutination reagent (Difco, Detroit, Mich.). Strains positive for group C antigen were graded as to size of colonies and zones of hemolysis. Strains were further characterized by Voges-Proskauer tests and sugar fermentation reactions as previously described (22). The streptococci were preserved by freezing in skim milk and storage at −70°C. Fifteen of the strains were isolated from patients with acute pharyngitis, and five control strains were from patients with noninfectious problems.

Mouse passage. In some experiments group C streptococcal strains were passed in mice to enhance M-protein expression (30, 31). Mice were inoculated intraperitoneally with approximately 10^6 CFU of streptococci. Eighteen to 24 h later they were euthanized, and the spleen was removed aseptically and emulsified in a small volume of Todd-Hewitt broth with a mortar and pestle. An aliquot of the emulsified tissue was inoculated on a sheep blood agar plate and incubated aerobically at 37°C overnight. Isolated colonies of group C streptococci were harvested for use in studies of phagocytic killing.

Growth in human blood. The technique employed for growth has been published in detail elsewhere (9) and serves as an assay for expression of functional M protein (7, 12). Log-phase cultures of group C streptococci in Todd-Hewitt broth were serially diluted in phosphate-buffered saline (pH 7.4) in 10-fold increments. One-tenth-milliliter aliquots of the diluted cultures containing approximately 1 to 200 CFU were inoculated into four plastic tubes (Falcon; Becton Dickinson Laboratories, Lincoln Park, N.J.) containing 0.3 ml of lightly heparinized (10 U/ml) freshly drawn normal human blood. The tubes were rotated end-over-end for 3 h at 37°C. Quantitative pour plates were prepared from aliquots of the inocula and the rotated tubes. Plates in which the inoculum grew to confluence (colonies too numerous to count) were arbitrarily scored as 2,000 CFU. The ratio of the number of CFU inoculated to the number present in the phagocytic mixture at the end of incubation was determined. The $\log_2$ of this ratio represents the generations of growth, i.e., the average number of replications of the inoculum during the incubation period. Thus, an eightfold increase in the inoculum during incubation represents three generations of growth.

Southern hybridization. Southern hybridization was performed as previously described (17), using DNA purified from whole group C streptococcal cells by the method of Cleary et al. (16) and a gene probe isolated from pBR41-L3, a recombinant plasmid encoding the group A streptococcal M24 protein gene (*emm24*). pBR41-L was a gift of R. W. Baird and J. Dale, University of Tennessee, Memphis.

DNA sequence analysis. Sequence analysis was performed as previously described (17). Oligonucleotide primers homologous to the M-protein-encoding gene (*emmG1*) from a group G streptococcal strain (17) were used to amplify the region of the group C M-protein gene encoding the 5′ portion of the molecule encompassing the variable region. This amplified fragment was ligated to the vector pT7Blue (Novagen, Inc., Madison, Wis.), and the DNA sequence of the ends of the fragment was determined by the dideoxy chain termination method of Sanger et al. (37) as modified for use with Sequenase (U.S. Biochemical).

Nucleotide sequence accession numbers. Group C *emm* sequences have been submitted to GenBank under the following accession numbers: U65595 and U65596.

RESULTS

Resistance to phagocytosis in human blood. Nineteen of the 20 strains survived and multiplied to various degrees during the 3-h incubation period in normal human blood (Fig. 1). Eighteen (90%) grew vigorously, undergoing approximately five to eight generations of growth (i.e., 32- to 256-fold increases over the inoculum). Strain 435 initially achieved only two generations of growth, but its ability to resist phagocytosis was markedly enhanced by mouse passage (Fig. 1), a maneuver known to enhance expression of M protein. Only 1 of the 20 strains, i.e., 1015, isolated from an asymptomatic control, failed completely to resist phagocytic killing both before and after mouse passage.

Southern hybridization. The DNA probe corresponded to the carboxy-terminal one-third of the group A M-protein gene *emm24*. This region is highly conserved in all M proteins studied thus far. Genomic DNA from all 20 strains showed homology to the probe under stringent hybridization conditions. Results for nine of the strains are shown in Fig. 2.

Terminal DNA sequences of group C M-protein genes. The DNA sequences of the 5′ regions of the M-protein-encoding genes from three group C strains were determined, and from these sequences the N-terminal amino acid sequences were predicted. Two of the group C strains (839 and 279) were from patients with acute pharyngitis, and the third (1139) was from an asymptomatic control.

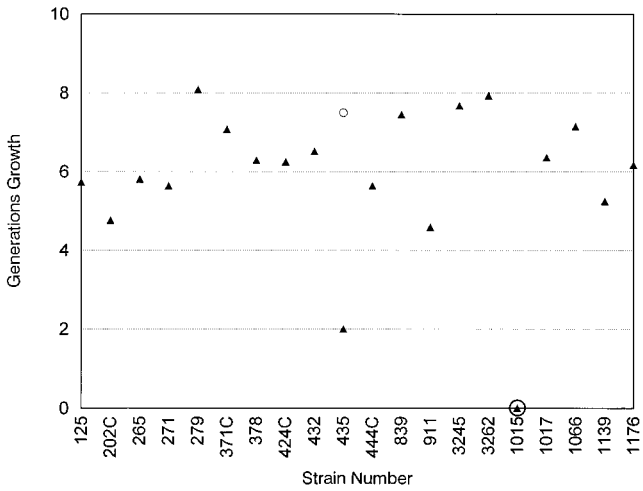

FIG. 1. Generations of growth of pharyngeal strains of *S. equisimilis* in human blood. Generations of growth indicates the number of replications of the inoculum during the incubation period (see Materials and Methods). Strains 1015, 1017, 1066, 1139, and 1176 (far right) are from asymptomatic patients; all other strains were isolated from patients with acute pharyngitis. Open circles denote growth of selected strains after mouse passage.

The DNA sequences indicated that the two disease-associated isolates have identical M-protein genes and that these genes differ from the M-protein gene of group C strain 1139. The predicted amino acid sequence suggests that the M proteins synthesized by group C strains 839 and 279 have the 41-amino-acid signal peptide MARKNTNKHYSLRKLKTGT ASVAVALTVVGAGLASQTEVKA. The first eight amino acids of the signal peptide are identical to the first eight amino acids of the group G MG1 signal peptide. This is to be expected, however, since the oligonucleotide primers used for

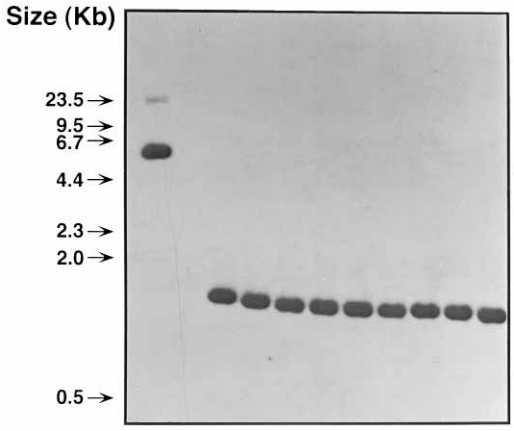

FIG. 2. Genomic DNA from *S. equisimilis* strains was digested with *Hin*dIII and hybridized under stringent conditions with the *emm24* gene probe. All strains showed a sequence homologous to that of the gene probe. Lanes: 1, *Hin*dIII-digested pBR41-L3 (*emm24*-encoding plasmid); 2, no specimen; 3, group C strain 3262; 4, strain 1176; 5, strain 378; 6, strain 279; 7, strain 1139; 8, strain 432; 9, strain 1017; 10, strain 202C; 11, strain 1015.

the PCR correspond to this region. The next 33 amino acids of the group C signal peptide are 97% identical to the next 33 amino acids of the group G MG1 signal peptide. In addition, this signal peptide is 98% identical to a predicted signal peptide from a previously reported group C M protein (GenBank accession number X60097).

The predicted N terminus of the 839 and 279 M protein is DHPRVENARNEVLKYQYVPAAQ. This sequence was only slightly related to the N termini of the M protein from strain 1139. However, it was 88% identical to the amino acid sequence of the M protein from a group G streptococcal strain, LG496 (GenBank accession number X79525). This suggests that certain group C and group G streptococcal strains may contain identical M-protein genes.

The predicted signal peptide from group C strain 1139 was 90% identical to the group G MG1 signal peptide and 92% identical to the M-protein signal peptide from group C strains 839 and 279. If the signal peptide cleavage sites are identical between the group G and the group C M proteins, then the amino-terminal sequence of the strain 1139 M protein is NEEVTGRLVLLVGRA. This sequence is unique to this protein, and there are no proteins with identical or nearly identical sequences in the GenBank database. The partial DNA sequences of regions of the M-protein genes encoding the C repeat regions from all three group C streptococcal strains indicate that they contain the class I epitopes that characterize M proteins of rheumatogenic serotypes of group A streptococci (4–6).

DISCUSSION

Group C streptococci have caused well-documented epidemics of acute pharyngitis, often associated with contaminated foods. Two such epidemics due to milk contaminated with *Streptococcus zooepidemicus* were associated with the development of poststreptococcal acute glomerulonephritis (1, 19). Not all outbreaks of group C streptococcal pharyngitis, however, have been associated with a common source. During a 3-month period Benjamin and Perriello (3) isolated group C streptococci from the throats of 32 students at a school for boys with learning disabilities. Children with positive throat cultures had significantly higher mean serum antistreptolysin O titers than did those with negative cultures, and 22 (69%) of the students with positive cultures exhibited signs and symptoms of acute pharyngitis.

Although the examples cited above document the capacity of group C streptococci to cause acute pharyngitis under special epidemiologic circumstances, there remains a question as to the role of these organisms as a cause of symptomatic sore throat in endemically occurring pharyngitis in open populations (15, 25). Group C streptococci are frequently isolated from asymptomatic individuals, particularly adults, and prospective studies have not always shown clear-cut differences in isolation rates between symptomatic and asymptomatic individuals. Recent data, however, provide evidence for the role of these organisms in community-acquired pharyngitis. Meier et al. (33) isolated group C streptococci more frequently from 1,425 adults with sore throat than they did from 284 controls (6% versus 1.4%). Moreover, the signs and symptoms of group C-associated pharyngitis were intermediate between those of group A streptococcal pharyngitis and those of culture-negative pharyngitis. In a study of college students, Turner et al. (47) found group C streptococci to be isolated significantly more frequently from patients with acute pharyngitis than from those with noninfectious problems (26% versus 11%). Among patients with sore throat, those with positive cultures for group C streptococci were significantly more likely to have fever, exudate, and anterior cervical adenopathy than were those with negative cultures. Additional studies with this student population demonstrated that students from whom *S. equisimilis* was isolated had clinical features more suggestive of pyogenic infection and higher colony counts on primary culture plates than did those from whom *S. anginosus* was recovered (46).

These observations, plus the well-documented propensity of group C streptococci to cause bacteremia and life-threatening invasive disease in humans (2, 11, 13, 14, 29, 35, 39, 42, 43), prompted the present study. Because of the preeminent role of M protein in virulence of group A streptococci, we directed our attention to this molecule.

M protein exhibits an alpha-helical coiled-coil structure. Its C-terminal portion, which is anchored to the cell membrane and traverses the cell wall, is highly conserved, whereas the N-terminal portion, which is in contact with the external environment, is highly variable and accounts for type specificity. There are more than 80 known M-protein serotypes of group A streptococci, and type-specific antibodies to them are believed to be responsible for acquired human immunity. M proteins similar in structure and function to those of group A streptococci have been detected in group G streptococci (8, 17, 28, 38). Although certain group G strains express an M protein identical to that of group A M12 (32, 40), others appear to represent unique M types.

The data indicate that all *S. equisimilis* strains isolated from the throats of our patients possess genomic DNA encoding the conserved portion of type 24 M protein. Furthermore, nearly all of the strains are highly resistant to phagocytic killing in fresh human blood, suggesting that functional M protein is expressed on the bacterial surface. The fact that four of the five strains isolated from asymptomatic individuals multiplied luxuriantly in human blood is not surprising, because the initiation of clinical infection may well be dependent upon local and systemic modalities of host defense (including the presence of type-specific antibody) rather than exclusively upon virulence properties of the colonizing strain.

M proteins have previously been detected in equine strains of *S. equi* (10, 23, 27) and *S. zooepidemicus* (45). The proteins elicited protective antibodies and impaired deposition of C3 on the bacterial surface (10). The M-protein gene of an equine *S. zooepidemicus* strain has been cloned (45). The predicted amino acid structure contained an alpha-helical repeat and showed considerable homology to the carboxy termini of M proteins of group A and group G streptococci, but the extensive A, B, and C repeat regions found in group A M proteins were absent.

Less is known regarding M proteins of large-colony (*S. equisimilis*) organisms involved in human infections. Podbielski et al. (34), utilizing PCR assays and genomic fingerprinting, detected *emm* genes in 75% of 28 group C strains isolated from human specimens in Germany and the United States. Neither the group C species nor the bodily site of isolation was identified. The authors' findings, however, were in agreement with our own, in that the predicted structures of the M proteins were highly homologous to those of M proteins of group A streptococci in the C repeat (conserved) region but the N terminus was unique. Efstratiou et al. (21) utilized conventional microbiologic (resistance to phagocytosis) and immunologic (type-specific opsonization and precipitation) techniques to identify M proteins in strains of *S. equisimilis* isolated from four outbreaks of human disease (21, 24). Two of the outbreaks were associated with pharyngitis, and the strains from

these outbreaks (one British and one Romanian) belonged to different M types.

The predicted amino acid sequences of the N-terminal (variable) portions of our isolates indicate that certain strains of *S. equisimilis*, such as 839 and 279, express M proteins related or identical to those of group G streptococci. This finding is compatible with the previously suggested hypothesis that M proteins may be acquired by horizontal transfer across serogroups (40). On the other hand, the fact that the predicted N-terminal amino acid sequence of strain 1139 has not been encountered before suggests that it may represent a new M type. Further studies will be required to determine whether *S. equisimilis* strains express multiple unique M antigens, as do strains of groups A and G (34, 38).

It is of interest that the M proteins of both group C and group G streptococci (17) possess the surface-exposed, conserved region domain common to M proteins of group A types epidemiologically associated with rheumatic fever (4, 5). Although the M proteins of *S. equisimilis* and large-colony group G streptococci are remarkably similar in both structure (17) and function (8, 12) to those of rheumatogenic group A streptococci, the former organisms have never been found to cause acute rheumatic fever. This fact suggests that if M proteins are involved in the pathogenesis of rheumatic fever, they are not in themselves sufficient to elicit this immunologically mediated disorder.

The studies presented here are, to our knowledge, the first demonstration by genetic and functional techniques of M proteins in strains of *S. equisimilis* isolated in cases of endemically occurring acute pharyngitis. These studies do not establish a pathogenic role for this microorganism in pharyngitis. They do add additional substance, however, to the clinical and epidemiologic data summarized above. Investigation of the group A streptococcal strains involved in acute rheumatic fever, poststreptococcal glomerulonephritis, and the streptococcal toxic shock syndrome has revealed that the clinical consequences of a given streptococcal infection are highly strain dependent. This is likely also to be true for group C streptococci and may explain conflicting opinions regarding the pathogenic potential of *S. equisimilis*. A better understanding of the role of M proteins and other factors governing strain virulence may clarify the situation.

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