Mechanisms of disease

Streptolysin S and necrotising infections produced by group G streptococcus

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Summary

Background We encountered three patients with severe necrotising soft tissue infections due to β-haemolytic group G streptococcus. Due to strong clinical similarities with invasive infections produced by group A streptococcus, we investigated a potential link of shared β-haemolytic phenotype to disease pathogenesis.

Methods Hybridisation, DNA sequencing, targeted mutagenesis, and complementation studies were used to establish the genetic basis for group G streptococcus β-haemolytic activity. The requirement of group G streptococcus β-haemolysin in producing necrotising infection was examined in mice.

Findings Each patient had an underlying medical condition. β-haemolytic group G streptococcus was the sole microbial isolate from debrided necrotic tissue. The group G streptococcus chromosome contained a homologue of the nine-gene group A streptococcus sag operon encoding the β-haemolysin streptolysin S (SLS). Targeted mutagenesis of the putative SLS structural gene sagA in group G streptococcus eliminated β-haemolytic activity. Mice injected subcutaneously with wild-type group A streptococcus or group G streptococcus developed an inflammatory lesion with high bacterial counts, marked neutrophil infiltration, and histopathological evidence of diffuse tissue necrosis. These changes were not found in mice injected with the isogenic group A streptococcus or group G streptococcus SLS-negative mutants.

Interpretation In patients with underlying medical conditions, β-haemolytic group G streptococcus can produce necrotising soft tissue infections resembling those produced by group A streptococcus. The β-haemolytic phenotype of group G streptococcus is produced by the exotoxin SLS, encoded by a functional homologue of the nine-gene group A streptococcus sag operon. SLS expression contributes to the pathogenesis of streptococcal necrotising soft tissue infection.

Introduction Group G streptococci are commonly part of the normal flora of human skin, pharynx, and gastrointestinal tract.1 Human group G streptococcus isolates are subdivided on the basis of colony size and haemolytic phenotype on sheep blood agar. Small colony group G streptococcus exhibit variable haemolytic reactions and are classified within the Streptococcus milleri group. Large colony group G streptococcus isolates, now classified as S dysgalactiae subspecies S equisimilis,2 produce robust β-haemolysis and are morphologically very similar to the prominent pathogen group A streptococcus.

Since the mid-1980s, an increase in life-threatening invasive infections produced by group A streptococcus has been well documented.3,4 Prominent among these syndromes is necrotising fasciitis, a destructive

Panel 1

Case 1: A 52-year-old man with type II diabetes mellitus was admitted after 6 days of fever and right leg swelling. Oedema and erythema extended from knee to ankle with tender right inguinal adenopathy. The patient received intravenous cefazolin and clindamycin but remained febrile. He developed lymphangitic streaking of his thigh and bullae on his leg. Cultures of blood and bulblous fluid were negative. On day 3 the patient underwent surgical debridement. The fascia of his right medial calf was grossly thickened and necrotic, and histopathology revealed extensive acute necrotising inflammation and intravascular thrombosis (figure 1). Cultures from the fascial tissue grew group G streptococcus. He was changed to intravenous benzylpenicillin for a 4 week course. The patient required two additional surgical debridements but improved and was discharged to a rehabilitation facility.

Panel 2

Case 2: A 59-year-old man with hairy-cell leukaemia and neutropenia presented with fever and left calf pain. On examination the calf was mildly oedematous but tender to palpation. Full blood count showed white blood cells 0·5×10⁹/L with absolute neutrophil count 0·04×10⁹/L, haemoglobin 125 g/L, and platelets 74×10⁹/L. He was admitted and treated with piperacillin and tobramycin. Many blood cultures grew group G streptococcus and antibiotic therapy was changed to benzylpenicillin. Due to continuing pain, calf tenderness, and erythema magnetic resonance imaging was done on day 7, showing a 10×7 cm fluid collection within the soleus muscle. Irrigation and debridement of the abscess and surrounding muscle tissue was done. Intraoperative cultures grew group G streptococcus and histology was consistent with myonecrosis. He was treated with intravenous penicillin for 4 weeks and granulocyte-colony stimulating factor. He improved on this regimen with full recovery after rehabilitation.
infection of the subdermal soft tissues frequently complicated by toxic shock syndrome. By contrast, serious group G streptococcus infections occur only rarely, including endocarditis, septic arthritis, bacteremia, and septic shock. We identified only one published case each of necrotising fasciitis or myositis caused by group G streptococcus.

Here we report three cases of severe necrotising infections due to \( \beta \)-haemolytic group G streptococcus (panels 1, 2, and 3). Because of similar clinical presentations to group A streptococcus infections, we investigated a link between bacterial \( \beta \)-haemolysin phenotype and disease pathogenesis. We used molecular techniques and a murine infection model to identify the \( \beta \)-haemolysin of pathogenic human group G streptococcus, and assess its contribution to disease pathogenesis.

**Methods**

Group G streptococcus isolates were identified by the API 20 Strep identification system (bioMérieux, St Louis, MO, USA). Published methods were used for M-protein \( (\text{emm}) \) genotyping, T-antigen typing, opacity factor testing, and \( \text{PULSED-FIELD GEL ELECTROPHORESIS (PFGE)} \) analysis. Haemolytic titres were determined in a liquid-phase assay in aerobic growth conditions. We used culture and \( \text{TRANSFORMATION} \) conditions as previously described.

The group G streptococcus isolate from Case 1 (VASD1) was selected for genetic and animal virulence studies. We did dot-blot hybridisation analysis with digoxigenin-labelled group A streptococcus \( \text{sag} \) gene probes from the nine-gene \( \text{OPERON} \) encoding the \( \beta \)-haemolysin streptolysin 5 (SLS). A 2.4 kb \( \text{HindIII} \) fragment of group G streptococcus chromosomal DNA probe that was positive for \( \text{sagA} \) by
Southern blot analysis was cloned in Escherichia coli and sequenced directly.

For mutagenesis studies, we amplified an intragenic fragment from the group G streptococcus saga gene using PCR and cloned in temperature-sensitive vector pHY304. This knockout plasmid was introduced into group G streptococcus by electroporation, and transformants selected at 30°C. Homologous recombination events in the group G streptococcus chromosome were identified by shifting to the non-permissive temperature (37°C) while maintaining antibiotic selection. Fidelity of site-directed recombination event was confirmed by PCR. Complementation was done as follows: the group G streptococcus saga knockout mutant was rendered competent and transformed with vector pSagLocus containing the SLS operon genes of group A streptococcus.

We tested group G streptococcus virulence in a mouse model of streptococcal necrotising fasciitis. Briefly, 10⁶ cfu of log-phase bacteria were mixed with Cytodex beads (Sigma) and injected subcutaneously into the right flank of hairless 4-week-old male Crl:SKH1(hrhr) Br mice (Charles River, Wilmington, MA, USA). Six animals were tested for the group G streptococcus parent strain and its isogenic saga mutant; group A streptococcus strain NZ131 (M49) and its corresponding saga mutant were tested for comparison. Animals were monitored for development of necrotic ulcers, and killed at 24 h or 48 h for quantitative culture and histopathological assessment. Data were compared using the exact Wicoxon rank-sum test.

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The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing the report.

Results

Each group G streptococcus isolate from the patients was S dysgalactiae, subspecies Equisimilis, with T-antigen type 25 and negative opacity factor. Group G streptococcus isolates from Cases 1 and 2 possessed M-protein (emm) gene stg480 (GenBank Accession number X79520) previously identified in group G streptococcus isolates from bloodstream and wound infections. Group G streptococcus isolates from Case 3 possessed the emm gene stc74a (GenBank Accession number X60097) which has been found in various β-haemolytic human group C streptococcus and group G streptococcus isolates. PFGE analysis showed that the group G streptococcus from Cases 1 and 2 were clonal, whereas the isolate from Case 3 was genetically distinct. No direct or common-source epidemiological contacts among the three patients were identified. The in-vitro haemolytic activities of the three invasive group G streptococcal isolates (mean titre 149 HU) were similar to those of six group G streptococcal strains isolated locally from epithelial sites (147 HU), and greater than a panel of 13 group A streptococcal strains of differing M-protein serotype provided by the Centers for Disease Control (23 HU).

We examined chromosomal DNA from strain VASD1 (from patient 1) and four other β-haemolytic group G streptococcus strains for homologies to the nine genes (saga–sagI) in the group A streptococcus operon encoding SLS activity. By means of dot-blot analysis, strong hybridisation signals were obtained from group G streptococcal DNA with probes for each of the nine group A streptococcal genes (data not shown). DNA sequencing showed that the saga operon promoter and a terminator motif responsible for differential transcription of saga versus sagB-I were highly conserved. Comparison of predicted protein sequences for the group G streptococcus and group A streptococcus gene products showed 89% identity and 94% similarity for Saga (figure 2), 81% identity and 92% similarity for SagB, and 74% identity and 81% similarity for the N-terminus of SagC, respectively. Confirmed plasmid integral mutagenesis of saga yielded a group G streptococcus with no detectable β-haemolytic activity on SBA (figure 3). β-haemolytic activity could be partially restored to the group G streptococcus saga mutant by introduction of the intact group A streptococcus saga locus on a plasmid vector (figure 3). These studies show that a functional homologue of the group A streptococcus SLS (sag) operon is present in group G streptococcus. The new group G streptococcal sequence information has been submitted to the DDBJ/EMBL/GenBank databases under the accession number AY033399. We did additional sequencing of the saga gene on a group C streptococcal bloodstream isolate (T107) from the Toronto Invasive Bacterial Diseases Network (TIBDN) collection. This strain had a gene 100% homologous to saga from group G streptococcus patient isolate VASD1. Like group G streptococcus, large-colony group G streptococcus exhibiting robust β-haemolysis are classified within S dysgalactiae subspecies Equisimilis.

Figure 2: Comparison of the deduced amino-acid sequences of Saga between group G streptococcus (GGS) and group A streptococcus (GAS)

Figure 3: Elimination of group G streptococcus β-haemolysis by mutation of saga and partial complementation of the mutant phenotype upon transformation with the homologous group A streptococcus saga genes
The contribution of group G streptococcus SLS expression in the pathogenesis of necrotising fasciitis was tested in mice (table). Within 24–48 h, animals injected subcutaneously with the group G streptococcal clinical isolate developed necrotic ulcers at the site of inoculation, had high bacterial counts on lesion culture, and showed histopathological evidence of diffuse skin and soft tissue necrosis with substantial neutrophil infiltration. By contrast, mice injected with the group G streptococcus $sagA$ mutant did not develop necrotic ulcers, had ten-fold lower bacterial counts on lesion culture ($p=0.0022$), and showed minimal degrees of tissue injury or neutrophil infiltration. Representative gross and microscopic pathological findings are shown in figure 4. The results were similar to those seen with the group A streptococcus M49 strain and $sagA$ mutant used as a control and to our previously reported observations in M1 strains and SLS-negative transposon mutants.6 Two mice infected with the group G streptococcus $sagA$ mutant appeared to have cleared the infection by 48 h (<10 cfu/gm tissue). In the four mice without necrotic ulcers but with persistence of bacteria at the inoculation site, up to 20% of the recovered colonies had reverted to the wild-type $\beta$-haemolytic phenotype. The latter finding suggests an in vivo selective pressure toward excision of the integrative plasmid through reverse homologous recombination.

**Discussion**

We report three patients with necrotising soft tissue infections resembling group A streptococcus disease in which the sole microbial isolate was $\beta$-haemolytic group G streptococcus. A severe underlying medical condition was present in each case. Diabetes mellitus, malignancy, and cirrhosis are commonly reported risk factors for development of other types of invasive group G streptococcus infection.4,21,22 Despite initial therapy with intravenous antibiotics active against group G streptococcus, all three patients had clinical deterioration and grew viable organisms from the necrotic tissues when surgery was done. This observation reinforces the importance of prompt and thorough surgical debridement for the successful therapy of streptococcal necrotic fasciitis.4,5

The $\beta$-haemolysin of human pathogenic group G streptococcus and group C streptococcus is SLS, encoded by a nine-gene operon highly similar to that recently discovered in group A streptococcus.12 The
group G streptococcus SLS precursor, SagA, retains key features of this bacteriocin-type toxin, including a predicted Gly-Gly cleavage site to yield a propeptide matching the calculated size (2·8 kDa) of mature SLS. Downstream genes, including the putative modifying enzyme sagB and ATP-binding cassette exporter sagG-I are also conserved. Targeted mutagenesis of the group G streptococcus sagA gene abolishes β-haemolytic activity, and this phenotype is partially restored upon transformation of the mutant with the group A streptococcus homologue. These data confirm that genes of the sag operon are both necessary and sufficient for SLS production.

The bacteriocin-like SLS precursor SagA shares no homology whatsoever with streptolysin O (SLO), a 57-kD thiol-activated cytolysin expressed by group A, C, and G streptococci, for which the gene has been identified. By homology whatsoever with streptolysin O (SLO), a 57-kD production. Of notable exception are the absence in group G streptococcus of the group A streptococcus pyrogenic exotoxins SPE-A—the scarlet fever toxin A, and SPE-B, a chromosomally-encoded cytolytic protease. SPE-A production is strongly linked epidemiologically with strains identified in the present resurgence of invasive group A streptococcus infections. As we show with SLS, production of SPE-B, M-protein, and hyaluronic acid capsule are known to contribute to development of group A streptococcus necrotising fasciitis in the murine model. Absence of pyrogenic exotoxins or differences in the coordinate regulation of virulence factor expression may account for the apparent inactivity of group G streptococcus to produce necrotising fasciitis in the non-compromised host.

Strategies aimed at neutralisation of SLS activity could be of therapeutic benefit as adjuncts to definitive surgical and antibiotic management of streptococcal necrotising fasciitis.

**Contributors**

D Humar and V Nizet reported the clinical cases. V Datta, D Humar, DJ Bast, JCS De Azavedo, and V Nizet designed and carried out the molecular genetic and in vivo experiments. B Beall performed the emm-genotyping and PFGE analysis. D Humar, V Datta, and V Nizet prepared the original manuscript. All authors contributed to the revised manuscript. D Humar and V Datta contributed equally to this work.

**Conflict of interest statement**

None declared.

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