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 *sag*A 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.

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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 bullous 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 $0{\cdot}5\times10^9$/L with absolute neutrophil count $0{\cdot}04\times10^9$/L, haemoglobin 125 g/L, and platelets 74×10^9/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.

GLOSSARY

MUTAGENESIS
Creation of a heritable change in a specific DNA sequence.

COMPLEMENTATION
Restoration of phenotype to a bacterial mutant by reintroduction of an intact copy of the mutated gene on a plasmid vector.

OPERON
A set of genes which are grouped together and transcribed on the same messenger RNA.

HOMOLOGOUS RECOMBINATION
Substitution of a segment of DNA by another that is identical (homologous) or nearly so. Occurs naturally during meiotic recombination (crossing over); also used experimentally to modify the sequence of a target gene.

TRANSFORMATION
A process by which the genetic material carried by an individual bacterial cell is altered by incorporation of exogenous DNA on a plasmid vector or into its genome.

ISOGENIC MUTANT
A bacterial mutant differing from its wild-type parent strain by only a single genetic modification.

BACTERIOCIN
A small, naturally occurring protein produced by one species of bacterium that possesses antimicrobial activity against other bacteria. Some bacteriocins exhibit toxicity to eukaryotic cells.

TRANSPOSON
A relatively small DNA segment that has the ability to move from one chromosomal position to another, used experimentally for bacterial mutagenesis studies.

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)
A technique used to separate very large (megabase) DNA fragments for genotype analysis.

infection of the subdermal soft tissues frequently complicated by toxic shock syndrome.[5] By contrast, serious group G streptococcus infections occur only rarely, including endocarditis,[6] septic arthritis,[7] bacteraemia,[8] and septic shock.[9] We identified only one published case each of necrotising fasciitis or myositis caused by group G streptococcus.[10,11]

Here we report three cases of severe necrotising infections due to β-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 β-haemolysin phenotype and disease pathogenesis. We used molecular techniques and a murine infection model to identify the β-haemolysin of pathogenic human group G streptococcus, and assess its contribution to disease pathogenesis.[12]

Panel 3

Case 3: A 58-year-old homeless man with ethanol-induced cirrhosis and chronic lower extremity lymphoedema was admitted with a 3 day history of left thigh pain. Temperature was 35°C and blood pressure 70/50 mm Hg. His thigh was tensely swollen and erythematous from knee to groin with bullae formation. He was treated with cefazolin and clindamycin. He developed progressive acidaemia, hypotension, and coagulopathy. Intravenous immunoglobulin was administered. On day 3 he developed swelling and erythema of the left knee and left wrist, and numerous focal necrotic skin lesions of his digits. Gross examination of the left thigh fascia showed extensive liquefaction necrosis. Biopsy samples showed nectrotising fasciitis with contiguous myonecrosis. Septic arthritis of the left knee and left wrist were present, with fascial necrosis extending into the left extensor forearm. Extensive drainage and debridement were done. Blood, knee, wrist, thigh fascia, forearm fascia, and skin lesion cultures all grew group G streptococcus. Echocardiogram was normal. He developed adult respiratory distress syndrome, pneumonia, and candidaemia. He died on day 12 despite aggressive supportive care.

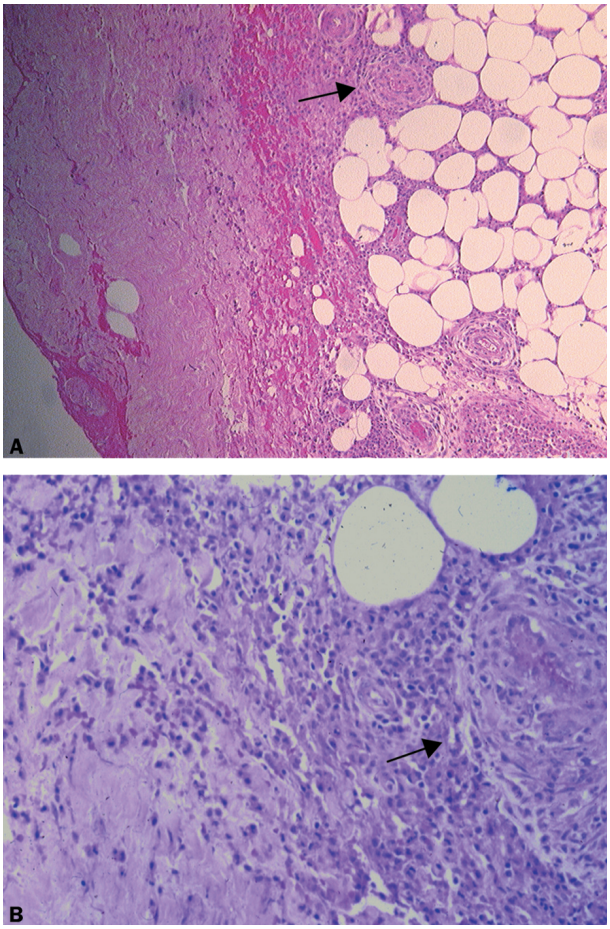

Figure 1: **Histopathology of debrided tissue from patient with group G streptococcal necrotising fasciitis of the calf**
Extensive tissue necrosis, vascular thrombosis (arrow), and neutrophilic infiltration can be seen on (A) low power (40x) and (B) high power (100x) views of the haematoxylin and eosin stained sections.

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 (*emm*) genotyping,[13] T-antigen typing,[14] opacity factor testing,[15] and PULSED-FIELD GEL ELECTROPHORESIS (PFGE) analysis.[16] Haemolytic titres were determined in a liquid-phase assay[17] in aerobic growth conditions. We used culture and TRANSFORMATION conditions as previously described.[12] The group G streptococcus isolate from Case 1 (VASD1) was selected for genetic and animal virulence studies. We did dot-blot hydridisation analysis with digoxigenin-labelled group A streptococcus *sag* gene probes from the nine-gene OPERON encoding the β-haemolysin streptolysin 5 (SLS). A 2·4 kb *Hind*III fragment of group G streptococcus chromosomal DNA probe that was positive for *sag*A by

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GGS: MLQFTSNILATSVAETTQVAPGG  CCCCCTTCCFSINVGGGSAQGGSGSYTPGK
     || ||||||||||||||||||||  |||||||||||| || |  |||||||||||
GAS: MLKFTSNILATSVAETTQVAPGG  CCCCCTTCCFSIATGSGNSQGGSGSYTPGK
     _______________________  ______________________________
          Leader peptide               Propeptide
     ________________________________________________________
                        Prepropeptide
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Figure 2: **Comparison of the deduced amino-acid sequences of *Sag*A between group G streptococcus (GGS) and group A streptococcus (GAS)**

Southern blot analysis was cloned in *Escherichia coli* and sequenced directly.

For MUTAGENESIS studies, we amplified an intragenic fragment from the group G streptococcus *sag*A gene using PCR and cloned in temperature-sensitive vector pHY304.[18] 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 *sag*A knockout mutant was rendered competent and transformed with vector pSagLocus containing the SLS operon genes of group A streptococcus.[12]

We tested group G streptococcus virulence in a mouse model of streptococcal necrotising fasciitis.[19,20] Briefly, 10^6 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 *sag*A MUTANT; group A streptococcus strain NZ131 (M49) and its corresponding *sag*A 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.

Role of funding source

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 (*sag*A–*sag*I) 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 *sag* operon promoter and a terminator motif responsible for differential transcription of *sag*A versus *sag*B-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 integrational mutagenesis of *sag*A 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 *sag*A mutant by introduction of the intact group A streptococcus *sag* 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 *sag*A 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 *sag*A 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*.[2]

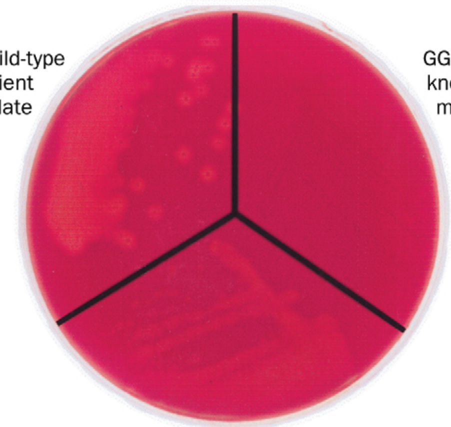

Figure 3: **Elimination of group G streptococcus β-haemolysis by mutation of *sag*A and partial complementation of the mutant phenotype upon transformation with the homologous group A streptococcus *sag* genes**

Bacterial strain tested	Necrotic ulcer formation (24 h)	Wound culture (cfu/gm) mean log value (24–48 h)	Wound culture (cfu/gm) range (24–48 h)	Neutrophilic infiltrate
Group G wild-type	100%	6·45	$1·9\times10^{6}$–$4·1\times10^{6}$	++++
Group G *sag*A mutant	0	3·87†	$1·0\times10^{0}$–$5·9\times10^{5}$*	+
Group A wild-type	100%	7·34	$1·3\times10^{5}$–$8·7\times10^{7}$	++++
Group A *sag*A mutant	0	4·93‡	$7·7\times10^{2}$–$1·2\times10^{5}$	+
Cytodex alone	0	NA	NA	+

Six mice were tested in each group. *2/6 mice had <10 cfu/gm recovered. †p=0·0022 *vs* wild-type. ‡p=0·005 *vs* wild-type. Exact Wicoxon rank-sum test.

Effect of streptolysin S gene mutation on group G streptococcus and group A streptococcus virulence in the murine model of necrotising fasciitis

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 *sag*A 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 *sag*A mutant used as a control and to our previously reported observations in M1 strains and SLS-negative TRANSPOSON mutants.[20] Two mice infected with the group G streptococcus *sag*A 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 β-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 β-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.[6,21,22] 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 nectrotic fasciitis.[4,5]

The β-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

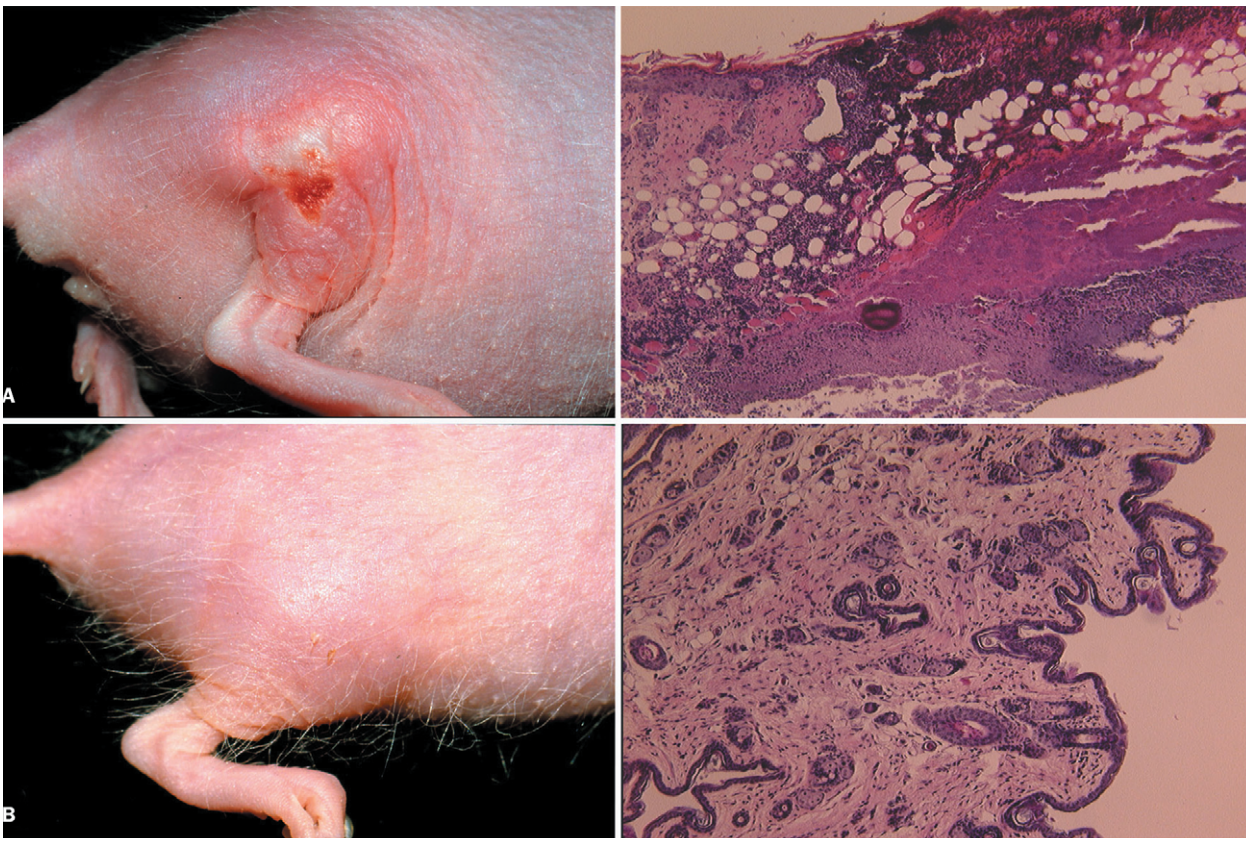

Figure 4: **Representative gross and microscopic histopathological findings in mice infected subcutaneously with a group G streptococcal necrotising fasciitis clinical isolate (A) versus an isogenic streptolysin S-deficient mutant (B)**
Ulcer formation with necrotic tissue destruction, vascular thrombosis, and diffuse neutrophilic infiltrate are noted with the wild-type strain, whereas only minimal inflammatory changes are seen with the mutant.

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 kD) of mature SLS.[23] Dowstream genes, including the putative modifing enzyme *sag*B and ATP-binding cassette exporter *sag*G-I are also conserved.[12] Targeted mutagenesis of the group G streptococcus *sag*A 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.[24] By contrast with SLS, SLO is inactivated by oxygen, inhibited by cholesterol, produces little to no detectable haemolysis on blood agar plates, is immunogenic, and oligomerises in the red-blood-cell membrane to form a pore. The β-haemolysin and virulence factor of the important human pathogen group B streptococcus is unrelated to SLS or SLO, but rather seems to be encoded by a new 78·3 kD gene, *cyl*E.[18,25] An interesting comparison for SLS may be the plasmid-encoded haemolysin/cytolysin expressed by 45–60% of *Enterococcus faecalis* (formerly Group D streptococcus) isolates from patients. Although sharing little primary sequence similarity to SagA, this small protein toxin is composed of two structural subunits that belong to the antibiotic class of bacteriocin peptides.[26]

In contrast to the wild-type parent strains, SLS-negative *sag*A mutants of group G streptococcus and group A streptococcus are not virulent in a murine model of streptococcal necrotising fasciitis. These findings suggest that SLS expression is required for the pathogenesis of this destructive infection. SLS is one of the most potent cytotoxins known,[27] capable of injuring a wide array of membranes including those of lymphocytes, neutrophils, and certain tissue culture cell lines.[28-30] The precise mechanisms of SLS membrane toxicity are not known. SLS does not seem to possess phospholipase action, and electron microscopic examinations of erythrocyte membranes damaged by SLS do not show large pores such as those induced by SLO.[31,32] We hypothesise that SLS contributes to the development of streptococcal necrotising fasciitis via direct toxicity to cells of the deep soft tissues and feeding vessels, leading to cell death and provoking neutrophil influx. In vitro and primate model studies with group A streptococcus suggest that neutrophil-derived reactive oxide metabolites and proteases may act together with bacterial cytotoxins to accelerate necrotic fascial injury.[33,34] SLS-mediated neutrophil lysis could cause release of such factors and prevent phagocytosis, explaining in part how wild-type group A streptococcus and group G streptococcus persist in the infection model despite intense neutrophil recruitment.

SLS toxin is clearly not sufficient to trigger necrotising fasciitis. SLS is produced by virtually all group G streptococcal and group A streptococcus isolates, even those isolated from asymptomatic individuals. Moreover, we found SLS haemolytic activity levels to be greater in group G streptococcus than the more virulent group A streptococcus. Human β-haemolytic group G streptococcus share many other important virulence factors with group A streptococcus including the antiphagocytic surface M and M-like proteins, streptokinase, fibronectin, and IgG binding proteins, SLO, C5a peptidase, NADase, and possibly a hyaluronic acid capsule.[1,35,36] 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 cysteine protease. SPE-A production is strongly linked epidemiologically with strains identified in the present resurgence of invasive group A streptococcus infections.[3,4] 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.[37,38] Absence of pyrogenic exotoxins or differences in the coordinate regulation of virulence factor expression[39,40] may account for the apparent inability 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 Dutta, 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.

Acknowledgments

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