Mitogenic factor (MF) is the major DNase of serotype M89 *Streptococcus pyogenes*

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To investigate the role of mitogenic factor (MF) in streptococcal pathogenesis, the structural gene (*mf*) encoding this protein was disrupted in a clinical isolate of *Streptococcus pyogenes* H293, to yield the isogenic mutant H363. Growth in enriched broth and on blood agar was unaffected by disruption of *mf*. Cell-free broth supernatants from H293 and H363 demonstrated identical promitogenic activities when co-incubated with human peripheral blood mononuclear cells, even when diluted 100 000-fold, showing that MF is not a major streptococcal mitogen compared with other secreted superantigens. Disruption of *mf* resulted in complete loss of DNase B production and detectable DNase activity in H363 compared with the parent strain, confirming that the single gene *mf*, which is present in all group A streptococcal M serotypes studied, encodes DNase B. Despite loss of DNase activity, the virulence of *S. pyogenes* in a mouse model of necrotizing fasciitis and myositis was unaffected.

Keywords: superantigen, nuclease, mouse model, necrotizing fasciitis

INTRODUCTION

The virulence of *Streptococcus pyogenes* is attributed both to an inherent resistance to opsonophagocytosis in non-immune whole blood and to a remarkable repertoire of secreted enzymes and toxins. Over the last decade, considerable attention has focused on the role of superantigens in streptococcal disease pathogenesis and toxic shock, in particular the ability of superantigens to cause clonal T cell proliferation and cytokine release (Herman *et al.*, 1991). Superantigens such as streptococcal pyrogenic exotoxins (SPEs) A and C are expressed only by phage-carrying strains, whereas the newly recognized structurally related streptococcal mitogenic exotoxin Z (SMEZ) and SPEG appear to be chromosomally encoded (Proft *et al.*, 1999). Mitogenic factor (MF, also termed SPEF) is a chromosomally encoded streptococcal toxin with apparently similar mitogenic properties to the above superantigens, though with quite distinct structure (Iwasaki *et al.*, 1993; Toyosaki *et al.*, 1996). It is well recognized that streptococci that lack the phage-encoded superantigens SPEA and SPEC can cause severe invasive disease with toxic shock (Davies *et al.*, 1996; Zurawski *et al.*, 1998); we therefore concluded

that chromosomally encoded virulence factors must account for the lethality of such strains.

Recombinant MF is reported to have nuclease activity and separate studies have shown that MF and the streptococcal nuclease DNase B share identical immunologic epitopes (Eriksson *et al.*, 1999; Iwasaki *et al.*, 1997), suggesting that the two proteins are equivalent. Antibodies to both DNase B and MF have been detected in patients recovering from streptococcal sepsis, showing that these proteins are synthesized by pathogenic streptococci during infection (Gerber *et al.*, 1980; Norrby-Teglund *et al.*, 1994a). Patients with toxic shock, however, have lower neutralizing antibody titres against MF than those with uncomplicated streptococcal disease, suggesting a pathogenic role for this protein (Norrby-Teglund *et al.*, 1994a). In this study, we set out to characterize the pathogenic role of MF in a *S. pyogenes* isolate which lacks the known phage-encoded superantigens, by disruption of the chromosomal copy of the gene *mf*.

METHODS

Bacteria, media and antibiotics. *S. pyogenes* strain H293 (M89, PT4245 typed by the Central Public Health Laboratory, Colindale, London) was isolated from thigh muscle tissue of a patient with necrotizing fasciitis and sepsis syndrome. Toxin

Abbreviations: IL-6, interleukin-6; MF, mitogenic factor; PBMC, peripheral blood mononuclear cells.

typing by PCR confirmed that the isolate was negative for *speA*, *speC*, *speH* and *ssa*, but positive for *mf*, *speG* and *smeZ*. PCR primers for *speC* and *ssa* were designed from the published gene sequences. PCR primers for *speA*, *mf*, *smeZ*, *speG* and *speH* have been previously described by Proft *et al.* (1999) and Unnikrishnan *et al.* (1999). Streptococci were grown in Todd-Hewitt (TH) broth with 0.2% yeast extract (THY; Oxoid) or on solid nutrient agar with 5% horse blood. In some cases, streptococci were grown in TH broth alone. *Escherichia coli* (Novablue cells; Novagen) were grown in Luria-Bertani (LB) broth or on solid LB agar. Kanamycin was used to select for transformants (400 µg ml⁻¹ for streptococci and 25 µg ml⁻¹ for *E. coli*).

Plasmids and transformations. A *Hind*III 346 bp internal fragment of the *mf* gene corresponding to nucleotides 943–1289 of the published sequence (Iwasaki *et al.*, 1993) was amplified from strain H305 using primers MF1, GCAAGC-TTCAAACACAGGTCTCA (forward), and MF2a, CGAAG-CTTCCGACATAAGACAGACC (reverse). The *Hind*III-cut product was cloned into the suicide vector pUCMUT1, a derivative of pUC19 which has the *aphA-3* kanamycin resistance gene flanked by two multi-cloning sites and no ampicillin resistance gene, to generate pUCMUT-mf (Sriskandan *et al.*, 1999). Plasmid DNA was then extracted from *E. coli* and used to transform strain H293 by electroporation as previously described by Caparon & Scott (1991) and Sriskandan *et al.* (1999), prior to kanamycin resistance selection. Transformation efficiency was standardized using a replicative control plasmid, pDL413, as previously described (Sriskandan *et al.*, 1999).

Southern hybridization and PCR. Targeted insertional disruption of *mf* caused by a single homologous recombination event between plasmid and chromosomal copies of *mf* was confirmed initially by PCR, using primer pairs annealing to streptococcal chromosomal DNA upstream of the structural *mf* gene (MFp, CTAGGTGACCACACAGCACC) and to the 3' region of *mf* distal to the intended region of homologous recombination (MF2, GCGAATTCGGTATAGCGCATGCCGCGC).

For Southern hybridization, parent strain and transformant genomic DNAs were digested with *Nde*I, *Hind*III and *Ssp*I, electrophoresed, then transferred to nylon, hybridized with the 346 bp digoxigenin-labelled *mf* fragment (amplified from H305 DNA using primers MF1 and MF2a) and developed with the DIG Detection kit (Boehringer Mannheim), prior to reprobing with *aphA-3* as previously described (Sriskandan *et al.*, 1999).

In vitro comparison of H293 and MF-negative mutant H363

Growth analysis of mutants. Growth of parent and mutant strains was compared by inoculating separate 100 ml volumes of THY broth with overnight streptococcal culture (5 ml) and monitoring OD₆₀₀ over 8 h, until stationary phase was reached. Cultures were diluted and plated onto blood agar to relate measured OD₆₀₀ to bacterial viable counts.

Promitogenic activity of H293 and H363. Human peripheral blood mononuclear cells (PBMC) from three normal volunteers were prepared by Ficoll density-gradient centrifugation (Pharmacia). Human PBMC were resuspended in tissue culture medium (RPMI 1640 with 10% FCS, 50 µg penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ and 2 mM glutamine) at 1 × 10⁶ cells ml⁻¹, and 198 µl suspension plated per well in 96-well plates (2 × 10⁵ cells per well). H293 and the transformant strain H363 were incubated at 37 °C overnight in tissue

culture medium without antibiotics. Both strains grew equally well based upon OD₆₀₀ and plating onto blood agar. Cell-free supernatants were filter-sterilized, subjected to 10-fold serial dilution and then co-incubated in 22 µl volumes with PBMC for 96 h. Final supernatant concentrations were therefore 1:10, 1:100, 1:1000, 1:10000 and 1:100000. Cell proliferation was measured by uptake of tritiated thymidine [1 µCi (37 kBq) per well in a volume of 20 µl] added 16 h prior to cell harvesting (Amersham Pharmacia Biotech).

DNase activity of H293 and H363. Cell-free supernatants (5 µl) from overnight antibiotic-free TH cultures containing H293 or H363 were inoculated into wells cut into DNase agar containing methyl green indicator dye (Oxoid). Aliquots (5 µl) of stationary-phase broth from H293 or H363 grown without antibiotic were streaked onto similar plates. Plates were then incubated for 5 h (in the case of supernatants) or 24 h at 37 °C and inspected for evidence of DNA hydrolysis.

Northern analysis of H293 and H363. RNA was prepared from THY broth cultures of H293 and H363, sampled at three different growth phases as previously described (Unnikrishnan *et al.*, 1999). RNA samples were run on agarose gel, blotted onto nylon, then hybridized with the 346 bp digoxigenin-labelled *mf* probe, prior to development using the ECL system (Amersham) as before.

Western analysis of supernatants from H293 and H363. For Western blotting, supernatants from overnight antibiotic-free TH broth containing H293 or H363 or TH alone were filter-sterilized. Streptococcal cultures demonstrated equivalent growth, based upon OD₆₀₀ and plating (H293, 1.4 × 10⁸ c.f.u. ml⁻¹; H363, 1.1 × 10⁸ c.f.u. ml⁻¹). Ten microlitres of each supernatant were subjected to SDS-PAGE under reducing conditions. The proteins were electroblotted onto nitrocellulose, which was then incubated successively with rabbit polyclonal anti-DNase B (1:10000 dilution, a kind gift of Dr Dieter Gerlach, Jena, Germany), biotinylated goat anti-rabbit IgG, streptavidin-conjugated horseradish peroxidase, and developed (ECL system; Amersham). Streptococcal DNase B enzyme reagent (5 µl of a 10× solution; Wampole Laboratories) was run as a positive control.

Comparison of H293 and H363 in vivo during sepsis

Bacterial preparation. H293 and H363 were grown overnight in 100 ml THY without antibiotic. Strains were washed and resuspended in up to 5 ml sterile saline, to give similar densities. For intramuscular infection, bacterial suspensions were adjusted to 2 × 10⁹ c.f.u. ml⁻¹ (approx.). Aliquots of each inoculum were diluted and plated out on blood agar to verify doses given.

Survival. Male outbred CD1 mice weighing 20–25 g (Charles River) were used in all experiments. Animals received food and water *ad libitum*. Two groups of 15 mice from the same batch (i.e. identical age and weight) received ~10⁸ c.f.u. H293 or H363 streptococcal suspension per mouse intramuscularly into the right thigh, as previously described (Sriskandan *et al.*, 1996). Mice were monitored over a 7 d period and those reaching the pre-determined end point were humanely killed. At 7 d, all surviving mice were killed; serum from cardiac puncture was frozen at –20 °C. The survival experiment was repeated once. All animal procedures were conducted within local and Home Office guidelines.

Bacteriology, histopathology and cytokine analysis. Three groups of five mice were infected with either H293 or H363 as above, or injected with saline and at 48 h after infection, mice were killed. Cardiac puncture was performed and tissues (right thigh, liver, spleen, kidney) were formalin-fixed,

Fig. 1. (a). Mutation strategy using circular plasmid pUCMUT-mf to transform wild-type (WT) *S. pyogenes* strain H293 chromosome, shown linearized. Black arrow on H293 DNA represents promoter for *mf* gene and black bar represents *mf* structural gene. Short black bar on pUCMUT-mf represents internal cloned segment of *mf* gene, and adjacent white bar represents the 1.5 kb *aphA-3* kanamycin resistance gene. A single cross-over event is shown between pUCMUT-mf and H293 chromosome, resulting in insertional mutation of the *mf* gene, shown in mutant H363. Note that insertion and duplication of plasmid pUCMUT-mf has occurred, separating native chromosomal *mf* structural gene from the promoter. Restriction sites relevant to Southern blot are marked. (b) Left panel: Southern hybridization of *NdeI*-cut genomic DNA from H293 and H363, probed with 346 bp *mf*, showing insertional mutation of the *mf* gene, corresponding to a 7 kb increase in restriction fragment size in H363 (2 × plasmid size). Right

sectioned and stained with haematoxylin and eosin prior to histopathological comparison. Portions (100 µl) of 10% diluted blood were plated directly onto blood agar for quantification of bacteraemia. Stability of mutation was confirmed by plating each *S. pyogenes* colony derived from H363-infected murine blood cultures onto both kanamycin and DNase agar. Furthermore, *S. pyogenes* colonies sub-cultured from H363-infected thigh muscle were replica-plated onto kanamycin blood agar plates. Sera from infected mice were frozen at -20°C prior to measurement of interleukin-6 (IL-6) levels by ELISA (R & D Systems).

Statistics. For *in vitro* experiments, the promitogenic effects of supernatants from parent strain H293 and MF-negative mutant H363 were compared by Mann–Whitney U test at each dilution tested. For *in vivo* studies, survival was recorded on Kaplan–Meier plots and groups were compared using the log rank test. IL-6 levels were compared using the Kruskal–Wallis and Mann–Whitney U tests. Values of $P < 0.05$ were considered significant.

RESULTS

Transformations

A single kanamycin-resistant transformant was obtained following electroporation of H293 with plasmid DNA; this was designated H363. H293 genomic DNA yielded an 800 bp product when subjected to PCR using primers MFp and MF2, whereas this product was absent when H363 genomic DNA was used as target. In contrast, genomic DNA from both strains yielded 450 bp and 350 bp products when primer pairs MF1/MF2 and MF1/MF2a, respectively, were used, as expected (not shown). These results suggested insertional disruption between the promoter region of *mf* and the structural *mf* gene. Southern hybridization following *NdeI* digestion of H293 genomic DNA using the 346 bp *mf* probe showed that the wild-type *mf* gene resided on a 1.1 kb restriction fragment, as predicted from the published sequence. *NdeI* was used as it does not cut within the *mf* gene or the plasmid pUCMUT-mf. The size of the *mf*-hybridizing fragment increased to 8.1 kb in H363, showing that 7 kb of DNA (exactly twice the size of the plasmid) had inserted into this fragment (Fig. 1a, b). Tandem insertion of the plasmid was confirmed by Southern hybridization using *HindIII*- and *SspI*-digested genomic DNA from H293 and H363, followed by probing with digoxigenin-labelled *mf* and *aphA-3* (Fig. 1b).

Growth analysis of H293 and H363

There were no detectable differences in colony size or morphology when H293 or H363 were cultured on blood agar, nor were there any differences in beta haemolysis when the two strains were cultured either aerobically or anaerobically (not shown). Both strains

panels: *HindIII*-cut and *SspI*-cut genomic DNA from H293 and H363 probed with 346 bp *mf* (upper panel) or *aphA-3* (lower panel), confirming tandem insertion of pUCMUT-mf at intended site.

Fig. 2. Growth of wild-type *S. pyogenes* H293 (●) compared with mutant H363 (○) in TH broth, measured as optical density at 600 nm.

Fig. 3. Promitogenic activity of increasing dilutions of RPMI supernatants from wild-type *S. pyogenes* H293 (black bars) or mutant H363 (hatched bars) co-incubated with human PBMCs for 96 h, measured by thymidine incorporation (c.c.p.m.; corrected counts per min). Each bar represents the mean of proliferation of PBMCs from three individual volunteers $\pm$ SD. Six separate cell incubations were performed for each individual at each dilution. No statistical differences between H293 and H363 were found either for individuals or the group (Mann-Whitney U test). Similar results were obtained after 72 h incubation in separate experiments with PBMCs from different donors.

grew comparably in THY broth; viable cell counting confirmed the results obtained by measuring optical density (Fig. 2).

Promitogenic activity of H293 and H363

Growth of streptococcal strains in RPMI tissue culture medium overnight did not differ between parent strain H293 (7.6×10^9 c.f.u. ml⁻¹) and H363 (4.3×10^9

Fig. 4. DNase activity of wild-type *S. pyogenes* H293 compared with mutant H363 on methyl green conjugated DNase agar. (a) DNA hydrolysis by supernatant from H293 but not H363 incubated at 37 °C for 5 h. (b) H293 and H363 cultured overnight at 37 °C, photographed on a lightbox. A clear zone of DNA hydrolysis is visible around the parent strain, but not the mutant. Results shown are representative of five separate tests.

c.f.u. ml⁻¹). The promitogenic activity of RPMI supernatants from H293 was not reduced following disruption of the *mf* gene in H363, even when supernatants were diluted 10^5 -fold (Fig. 3). Similar results were obtained in separate experiments where supernatants were co-incubated with PBMCs from different donors for 72 h (not shown).

DNase activity of H293 and H363

Cell-free supernatant from and culture of H293 caused a clear zone of DNA hydrolysis on methyl green DNase agar plates. In contrast, both supernatant from and culture of H363 failed to cause any detectable DNA hydrolysis (Fig. 4). Separate experiments failed to detect any DNase activity in supernatants from H363 at four different pH values (6.5–8.0), despite the fact that DNase activity was maintained in H293 even when diluted 50-fold. Quantitative estimation of DNase activity using the DNase enzyme reagent (Wampole Laboratories) in a colorimetric assay also failed to detect DNase activity in H363, despite maximal DNase activity in supernatant from H293 (not shown).

Fig. 5. (a) Upper panel: Northern analysis of RNA from wild-type *S. pyogenes* H293 and mutant H363 probed with 350 bp digoxigenin-labelled *mf* probe. Samples were obtained from three growth phases, mid-exponential (ME), late-exponential (LE) and stationary (ST). Molecular mass markers are shown on the right. Lower panel: ethidium-bromide-stained agarose gel prior to transfer of H293 and H363 RNA to nylon. (b) Western analysis of 10 µl stationary-phase supernatants from H293 compared with those from H363, immunoprobed with anti-DNase B anti-serum. TH, TH medium alone; M, low-molecular-mass markers; D, 5 µl commercial streptococcal DNase B. Bands corresponding to DNase B are seen in lanes D and H293 only.

Northern analysis of H293 and H363

Full length *mf* transcripts were seen in RNA samples from late-exponential and stationary-phase broth cultures of H293, but not in those of H363 (Fig. 5a). There was no evidence of a truncated mRNA transcript in RNA from mutant H363 (corresponding to the 5' 350 bp sequence of *mf* remaining on the disruption vector pUCMUT-*mf*).

Western analysis of H293 and H363 supernatants

Anti-DNase B antibody reacted with commercially available DNase B enzyme reagent, yielding a band of the expected molecular mass. Bands identical to these were detected in supernatants from stationary-phase cultures of H293, but not in those of H363 (Fig. 5b). As expected from the Northern analysis data, there was no evidence of a 12 kDa truncated protein product in broth from H363 (corresponding to the 5' 350 bp sequence of *mf* remaining on the disruption vector pUCMUT-*mf*), even when broths were concentrated fivefold (not shown).

In vivo infection experiments

Survival. In two separate experiments, survival of mice following *S. pyogenes* intramuscular infection was unaffected by disruption of the *mf* gene (Fig. 6). Infection with both H293 and H363 was characterized by marked swelling of the affected limb, with underlying supuration in the soft tissues. In the first experiment, 5/15 mice infected with H293 demonstrated skin loss with histological evidence of necrotizing cellulitis over the infected muscle; this was not seen in H363-infected mice. Skin loss was not seen in either group when the experiment was repeated.

Bacteriology. Blood cultures were drawn from five mice

in each group 48 h after onset of infection. 1/5 H293-infected mice was bacteraemic, whereas 3/5 H363-infected mice were bacteraemic (range 100–200 c.f.u. ml blood⁻¹). Thus there was no detectable diminution of systemic bacterial spread following disruption of the *mf* gene. All isolates were confirmed as group A streptococci. Blood isolates from H363-infected mice retained a kanamycin-resistant, DNase-negative phenotype when subcultured on appropriate agar. The frequency and level of bacteraemia measured 48 h after infection with either H293 or H363 was low compared with previous murine infections using different *S. pyogenes* strains (20). Purulent infected thigh muscle tissue obtained from surviving mice (four per group) on day 7 of infection was subcultured and shown to contain viable group A streptococci in pure culture. One hundred out of one hundred single colonies from four H363-infected mice demonstrated stable mutant phenotypes when subcultured onto kanamycin and were DNase-negative.

IL-6 levels. There were no significant differences in serum IL-6 levels between wild-type H293- and MF-negative mutant H363-infected mice ($n = 5$ mice per group). Mice infected with H293 had higher serum IL-6 levels (mean 1051 ± 369 pg ml⁻¹) compared with control mice injected with saline alone (mean 119 ± 167 pg ml⁻¹, $P = 0.008$). Mice infected with H363 had serum IL-6 levels of 731 ± 280 pg ml⁻¹; this was also significantly higher than saline controls.

Histopathology. Thigh muscle: a dense rim of neutrophils demarcated areas of central necrosis (including dead muscle cells and dead neutrophils) from healthy tissue in 5/5 mice from each group; no differences were observed between the two groups. Liver and spleen: no significant abnormalities were noted and no differences were observed between the two groups. Kidney: extensive tubular necrosis was observed in both groups of mice, associated with sloughing of epithelial cells into the

Fig. 6. Kaplan-Meier survival plots comparing survival of groups of 15 mice infected intramuscularly with either wild-type *S. pyogenes* H293 (○) or mutant H363 (●). Results of two separate experiments are shown. (a) Actual doses: H293-infected mice, 6.1×10^8 c.f.u. per mouse; H363-infected mice, 5.4×10^8 c.f.u. per mouse. $0.2 > P > 0.1$, log rank test. (b) Actual doses: H293-infected mice, 2.6×10^8 c.f.u. per mouse; H363-infected mice, 2.8×10^8 c.f.u. per mouse. $P > 0.2$, log rank test.

tubular lumen (casts), though glomeruli were preserved; these abnormalities were similar in extent in both groups.

DISCUSSION

Disruption of the *mf* gene in this *S. pyogenes* strain has demonstrated that the exotoxin MF, also known as SPEF, does not contribute significantly to the superantigenic activity of *S. pyogenes* when compared with other superantigens encoded by this strain, namely SMEZ and SPEG. Furthermore, we have shown that disruption of the gene encoding MF resulted in loss of DNase activity, associated with loss of DNase B production, confirming previous reports that the two proteins are the same. Finally, we have examined the importance of MF/DNase as a virulence factor in invasive streptococcal infection and have demonstrated that, at least in this strain, MF/DNase alone is not a critical virulence factor.

MF has been studied largely because of its reported superantigenic qualities. However, amongst the array of constitutive superantigens expressed by *S. pyogenes* strain H293, the mitogenic activity of MF was undetectable in the streptococcal supernatants used in our studies. Previously, we disrupted the gene encoding SPEA in *S. pyogenes* strain H305 and found a small but consistent diminution in promitogenic activity of the SPEA-negative mutant (Sriskandan *et al.*, 1999). In contrast, in this work, disruption of MF did not lead to any diminution of promitogenic activity. We considered the possibility that the parent strain H293 may not produce MF. However, separate transcription studies confirmed that *mf* mRNA was produced by H293 at a level similar to that seen in other strains (not shown). Furthermore, supernatants used in these studies were from stationary-phase cultures, a growth phase when *mf* transcription is known to be maximal (Unnikrishnan *et al.*, 1999) and proliferation studies were carried out at a time point when mitogenic effects of MF are thought to be maximal. We considered the possibility that the mutant H363 could produce a truncated MF product from the 350 bp sequence encoded by the disruption vector; this was discounted because no such product was detected at either RNA or protein levels. Norrby-Teglund *et al.* (1994b) showed that T cell proliferation

induced by purified MF was HLA class II-dependent and did not require conventional antigen processing, consistent with the definition of a superantigen. Subsequent proliferation studies using recombinant toxins have confirmed that recombinant (r) MF is mitogenic and can cause T cell proliferation compared with rSPEB, the streptococcal cysteine protease, in a class II-dependent TCR V β -restricted manner, similar to superantigens (Toyosaki *et al.*, 1996). However, in the same experiments 10^5 -fold more rMF was required to achieve levels of proliferation which were comparable with the recognized superantigens rSPEA or rSPEC. Although care must be taken when interpreting data from toxins expressed recombinantly, the data do suggest that MF lacks the potent T-cell-stimulating properties attributed to some of the other bacterial superantigens. Two studies have, however, shown that MF or rMF can induce significant cytokine production when co-incubated with human PBMCs, thus this secreted protein may yet contribute to the proinflammatory properties of *S. pyogenes* (Norrby-Teglund *et al.*, 1994c; Toyosaki *et al.*, 1996).

It has long been suspected that the genes encoding MF and DNase B are identical. A GenBank BLAST search using the sequence for MF published in 1993 (GenBank accession no. D13428) failed to locate registered sequences with significant homology. However, sequences listed under 1996 patent codes and annotated as streptococcal DNase B (GenBank accession no. A49208) demonstrate identity to *mf* when aligned. We considered the possibility that two virtually identical genes might co-exist in the *S. pyogenes* genome, though this was excluded in the wild-type strain we studied as only a single 1 kb fragment hybridized to the *mf* probe following *Nde*I genomic digestion. Identity at the protein level was first suggested by Iwasaki *et al.* (1997), who demonstrated that recombinant MF had DNase activity in addition to the mitogenic actions shown in earlier work. A subsequent study from Sweden showed that the two proteins were immunologically identical using polyclonal antisera (Eriksson *et al.*, 1999). Our study provides clear confirmatory evidence that a single structural gene within the streptococcal genome encodes the proteins MF and DNase B. Furthermore, both *mf* and DNase B sequences map to a single location in the now completed Oklahoma *S. pyogenes* M1 genome

database, confirming the identity of these genes in the this strain also (Suvorov & Ferretti, 1996).

We were initially surprised to find that DNase production was undetectable in the mutant strain H363, and considered the possibility that the insertional mutation induced in this study had resulted in a polar effect on other DNase-regulating genes adjacent to the target gene, or that the target gene formed part of a larger DNase-regulating operon. However, transcription studies have repeatedly shown that the transcript produced by the *mf* gene is monocistronic in this and other *S. pyogenes* strains and a polar effect or operon appears unlikely (Unnikrishnan *et al.*, 1999). We therefore conclude that, in the strain studied, DNase B is the only detectable DNase produced. *S. pyogenes* can produce four serologically distinct DNases, A–D, which, with the exception of DNase D (Podbielski *et al.*, 1996), have not been studied at the molecular level.

It is suggested that streptococcal DNase activity may facilitate liquefaction of pus and spread through tissue planes during invasive streptococcal infection (Bisno, 1995). Because of the evidence that MF might be involved in vascular permeabilization in sepsis and sero-epidemiological data which implicate MF as a virulence factor (Matsumoto *et al.*, 1999; Norrby-Teglund *et al.*, 1994a), we elected to compare the wild-type and isogenic MF/DNase-negative mutant strains in a mouse model of invasive necrotizing fasciitis. Disruption of *mf* did not, however, affect virulence of *S. pyogenes* with regard to host survival, histopathological change or bacterial growth during invasive infection and we have shown unequivocally that the induced mutation in strain H363 is rigorously stable *in vivo*. Podbielski *et al.* (1996) previously disrupted the DNase D gene in an M49 *S. pyogenes* strain and found opsonophagocytosis resistance unaltered, even though DNase activity was totally abrogated; *in vivo* studies of virulence were not reported. To our knowledge, this is the first study to address the specific role of a Gram-positive DNase in a sepsis model, though *Staphylococcus aureus* mutants deficient in a range of exoproteins including DNase are known to exhibit attenuated virulence (Bogni *et al.*, 1998). It is possible that DNase B has a more prominent role in different forms of infection or in throat colonization, or, indeed, in different streptococcal strains. The parent strain, H293, used in the current study induced a pattern of pyogenic change in murine thigh muscle plus severe tubular renal damage, which contrasts with more invasive changes seen previously with other *S. pyogenes* strains (Srisakandan *et al.*, 1996). It is noteworthy that mutation of the gene encoding MF was not achieved in an M1T1 *S. pyogenes* strain despite demonstrable bacterial cell competency (authors' unpublished observations); we cannot exclude the possibility that, in some strains, DNase activity might be essential. The development of a stable isogenic mutant lacking detectable DNase activity should facilitate studies to analyse the function of this group of enzymes. Furthermore, to avoid future confusion, a standardized nomenclature for this protein should be agreed upon.

ACKNOWLEDGEMENTS

This work was funded by a Medical Research Council (UK) Clinician Scientist award to S.S. (grant no. G108/245).

We are grateful to Dr Dieter Gerlach for providing purified protein and anti-DNase B serum, to Peter Fenwick for technical assistance in ELISA, and also to the University of Oklahoma *Streptococcus pyogenes* genome sequencing project for making the M1 sequence available on the Internet.

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Received 16 May 2000; revised 28 July 2000; accepted 3 August 2000.