BACTERIAL PATHOGENICITY

Role of certain virulence factors in a murine model of Staphylococcus aureus arthritis

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The susceptibility of male Swiss white mice (MF1) to Staphylococcus aureus-induced arthritis was investigated with wild-type strain allelic replacement mutants. Comparison was made with a known mouse arthritogenic strain. The development and severity of arthritis were dependent both on the numbers of live bacteria injected intravenously and also on the mutant used; the ID50 ranged from \((5 \times 10^6)\) to \((1 \times 10^8)\) cfu. The results indicate that expression of the genes associated with virulence, including those for protein A and \(\alpha\)-haemolysin, play a major role in the pathogenesis of staphylococcal septic arthritis. When either virulence component was carried by the \(S.\) aureus variant, a greater degree of inflammation, pannus formation and cartilage destruction was detected histologically. Loss of one or more virulence factors lowered the septic arthritis severity score based on clinical and histological parameters.

Introduction

The development of septic arthritis is a potentially life-threatening condition that requires prompt medical attention. Patients with rheumatoid arthritis are particularly susceptible to joint sepsis, which can result in a rapidly progressive and very destructive process [1]. Septic arthritis can develop following infection with any bacterial, fungal or viral pathogen [2]. The outcome depends on a number of factors, including the nature of the causative agent, the site of infection, the local or general resistance of the patient and the presence of any pre-existing joint abnormality [3]. Staphylococcus aureus is the most common cause of non-gonococcal bacterial arthritis [4] and accounts for some 80% of cases.

A murine model has been developed which results in the development of septic arthritis following a single intravenous (i.v.) injection of live \(S.\) aureus LS-1, initially obtained from a spontaneously swollen, arthritis joint in a mouse colony [5]. This experimental model allows the haematogenous spread of bacteria, which is usual in human septic arthritis. Previous experimental models in rabbits used the intra-articular route for the injection of bacteria; in one study 85 of 88 New Zealand White rabbits developed arthritic joints, two had non-swollen joints and one died [6]. However, the direct inoculation of bacteria into a joint cavity, as in this model, rarely occurs in man.

The aim of the present study was to develop a murine model of arthritis, which had previously been shown [5, 7] to develop following intravenous inoculation with viable \(S.\) aureus. The mice were inoculated i.v. through the tail vein with graded doses of \(S.\) aureus. The incidence of swollen joints was usually 50–60% with strain LS-1. Histological examination of the swollen joints demonstrated hypertrophy and proliferation of synovial tissue and pannus formation, with a mainly polymorphonuclear granulocyte infiltrate and smaller numbers of macrophages and fibroblasts; destruction of cartilage and subchondral bone was evident. Intraperitoneal, subcutaneous and i.v. inoculation of dead bacteria did not induce septic arthritis [8]. Bremell et al. extended their studies of this animal model by examining the immunological changes in infected mice; infection caused raised levels of tumour necrosis factor (TNF) and interleukin-6 (IL-6) within 24 h. Antibodies to cell wall components of strain LS-1 and to toxic shock syndrome toxin (TSST-1) were also detected, along with auto-antibodies which were predominantly of the immunoglobulin G isotype. These results favoured the notion of antigen-specific polyclonal B-cell activation during \(S.\) aureus arthritis [9].

We and others [9, 10] have tested other genotypes of
*S. aureus* in the same model. *S. aureus* strain NCTC 8325/4 (wild-type) and several mutants which had been derived by allele replacement [11] and varied in their expression of protein A, α- and β-haemolysins and coagulase were available. The accessory gene regulator (agr) is a polycistronic locus which regulates exoprotein synthesis in *S. aureus*. The majority of proteins which are agr-regulated are not synthesised or only at a considerably reduced rate in agr- mutants (e.g. the haemolysins), with the exception of the surface proteins whose expression in agr mutants [12] is increased. These mutants displayed decreased expression of extracellular toxins and enzymes but an enhanced expression of protein A and coagulase. Such agr- mutants were also used.

A variety of strains of *S. aureus* (LS-1 strain, NCTC 8325/4, allele replacement and agr- mutants derived therefrom) were examined in order to assess the role of virulence factors in the development of septic arthritis. Swiss white mice (MF1) were given a single i.v. inoculum of variable numbers of *S. aureus*. The mice were examined over 14 days and their joints were assessed both clinically and histologically. We have previously shown [13] that the expression or otherwise of protein A by these mutants directly affected their susceptibility to phagocytosis by human neutrophils. Protein A-positive strains were twice as resistant to ingestion by neutrophils as compared to their protein A negative counterparts. It was deemed important to ascertain whether mice responded similarly to such bacteria.

### Materials and methods

#### Bacterial strains

*S. aureus* LS-1 was kindly provided by Dr T. Bremell (University of Lund, Sweden) and its properties have been described previously [5]. Strain NCTC 8325/4 has been described previously and the mutants derived from it were provided by Dr T. J. Foster (Microbiology Department, Trinity College, Dublin) and described by his group [11]. Their genotypes are summarised in Table 1. The bacteria were cultured overnight on Columbia blood agar, prior to each experiment. The optical density of suspensions of the bacteria were measured spectrophotometrically at 620 nm and the bacteria were diluted in physiological saline to the required concentration. The *S. aureus* bacterial suspension was injected i.v. through the tail vein in a volume of 0.1 ml. Control animals were given physiological saline or formalin-killed bacteria (treatment with buffered formalin 4% for 4 h) in the same volume.

#### Evaluation of arthritis

The mice were examined individually at regular intervals for up to 14 days. Arthritis was defined as a visible swelling in the joints of either the fore or hind limbs. The overall appearance and behaviour of the animals was also noted. The joints were taken for histological examination in their entirety and preserved in buffered formalin 4%.

### Clinical scoring

Joint involvement was scored 1–3 on the basis of 1 point for mild swelling or erythema or both, 2 points for moderate swelling and erythema and 3 points for marked swelling and erythema and occasionally ankylosis. Such changes were assessed and scored for each limb and the mean score per animal group was taken.

### Histological examination

The joints were preserved in buffered formalin 4% then subjected to routine fixation, decalcification, paraffin embedding, sectioning and staining with haematoxylin and eosin. The histological scoring for each joint was based on the degree of joint damage and destruction and whether other organs were also involved. Scores were 1 point for mild, 2 points for moderate and 3 points for severe septic arthritis or osteomyelitis. Scoring was again taken as the mean per animal group.

### Results

The i.v. injection of various doses of *S. aureus* LS-1 strain showed that the mice became more susceptible to arthritis with larger bacterial inocula; however, mortality also increased (Fig. 1). The clinical appearance (listlessness, apatosis, ruffled coat and weight loss) of the mice indicated that the animals were septicemic. Histopathological examination of swollen joints re-

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>NCTC 8325/4</td>
<td>spa- hly+ hlb+ coa+</td>
</tr>
<tr>
<td>DU5720</td>
<td>spa- hly+ hlb- coa+</td>
</tr>
<tr>
<td>DU5722</td>
<td>spa- hly- hlb+ coa+</td>
</tr>
<tr>
<td>DU5723</td>
<td>spa+ hly+ hlb+ coa+</td>
</tr>
<tr>
<td>DU5814</td>
<td>spa+ hly- hlb+ coa+</td>
</tr>
<tr>
<td>DU5818</td>
<td>agr- spa- hly- hlb-</td>
</tr>
<tr>
<td>DU5821</td>
<td>agr- spa- hly+ hlb+</td>
</tr>
<tr>
<td>DU5852</td>
<td>spa- hly- hlb+ coa- cf</td>
</tr>
</tbody>
</table>

Gene coding: spa, protein A; hly, α-haemolysin; hlb, β-toxin; coa, coagulase; cf, clumping factor; agr, accessory gene regulator.
Fig. 1. Effect of various doses (cfu) of *S. aureus* LS-1 injected i.v. on the incidence of arthritis in Swiss white (MF1) mice: □, swollen joints; □, tail lesions; ■, mortality.

Control mice given the same volume of saline as in the bacterial inoculum did not develop swollen joints and no histological changes were apparent (Fig. 3). In addition, five animals per group were inoculated i.v. with either $1 \times 10^7$ or $1 \times 10^8$ formalin-killed bacteria. The animals were observed for 14 days with no clinical evidence of swollen joints. Histological examination revealed no joint destruction or abnormalities.

Having demonstrated some degree of reproducibility of the murine model, further investigations were performed with strain NCTC 8325/4 and its genetically engineered mutants. The mutants varied from the wild-type strain by one or more gene determinants for a virulence factor (protein A, α-haemolysin, β-haemolysin and clumping factor). The role of these factors in the development of murine arthritis was compared. Septic arthritis developed soon after injection with strains NCTC 8325/4, DU5814 and DU5852 (all producers of protein A) with clinical evidence of swollen joints apparent after 2 days. However, mutants DU5720 and DU5722 (both unable to produce α-haemolysin) failed to produce clinical signs of joint swelling until day 7 (Table 2). In most joints there was synovial hyperplasia and evidence of severe joint destruction. In some animals areas of inflammation and osteomyelitis with secondary involvement of bone were evident. Certain animals without clinically swollen joints were shown histologically to have joint destruction and septic arthritis, demonstrating that haematogenous spread of bacteria and seeding of *S. aureus* in the joint had taken place without visible signs of inflammation. The experiment was repeated with *agr* mutants of NCTC 8325/4. Two such mutants (DU5821 and DU5818), which expressed different amounts of protein A, failed to induce swollen joints until day 7. Use of the histological
scoring system revealed that most damage was caused by the wild-type strain and mutants DU5723 and DU5852, both of which produced α-haemolysin, and DU5818 which over-expressed protein A but failed to elaborate α-haemolysin. It would appear that some compensation for the absence of haemolysin expression in vivo is provided by the extra production of protein A by this variant.

Discussion

The present studies mirror those of Bremell et al. [5] who demonstrated almost no septicaemic phase in mice inoculated i.v. with $1 \times 10^7$ live *S. aureus* LS-1 cells. The study with NCTC 8325/4 showed a higher incidence of septicaemia and mortality, some tropism for the articular joints, and evidence of systemic spread.

Fig. 2. Histopathological appearance of murine septic arthritis 5 days following i.v. injection of $1 \times 10^7$ cfu of *S. aureus* LS-1 showing an acute inflammatory response within the synovium. H & E stain, × 400.

Fig. 3. Normal appearance of murine joint. H & E stain, × 400.
Table 2. Arthritis severity score and histological damage assessment at 14 days with different variants of *S. aureus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arthritis severity score</th>
<th>Histological damage assessment</th>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td>NCTC 8325/4</td>
<td>0.95</td>
<td>1.9</td>
<td>spa-hly~</td>
</tr>
<tr>
<td>DU5720</td>
<td>0.5</td>
<td>0.4</td>
<td>spa-hly~</td>
</tr>
<tr>
<td>DU5722</td>
<td>0.45</td>
<td>0.4</td>
<td>spa-hly~</td>
</tr>
<tr>
<td>DU5723</td>
<td>0.4</td>
<td>0.6</td>
<td>spa-hly~</td>
</tr>
<tr>
<td>DU5814</td>
<td>0.58</td>
<td>0.5</td>
<td>spa-hly~</td>
</tr>
<tr>
<td>DU5818</td>
<td>0.73</td>
<td>0.6</td>
<td>agr-hly-spa~</td>
</tr>
<tr>
<td>DU5821</td>
<td>0.68</td>
<td>0.7</td>
<td>agr-hly-spa~</td>
</tr>
<tr>
<td>DU5852</td>
<td>0.85</td>
<td>1.5</td>
<td>spa-hly<del>cf</del></td>
</tr>
</tbody>
</table>

Scores are based on several experiments with different batches of mice inoculated i.v. with $1 \times 10^8$ cfu of *S. aureus*. At least 10 mice/group were included in the assessments.

Several mutants of *S. aureus* have been shown to induce joint sepsis of varying severity in this animal model. The rapid onset of inflammation that occurred with the wild-type strain NCTC 8235/4 and with strain LS-1 has important implications for the development of joint sepsis in man. *S. aureus* has been shown to possess a number of genes that govern the expression of soluble virulence factors (α- and β-haemolysins), which are important in pathogenesis. The presence of these genes in the *S. aureus* mutant DU5723 and wild-type strain resulted in the early onset of arthritis, compared to the later development of similar symptoms in animals inoculated with the other mutants which did not express these toxins. Similarly, there was an early onset of arthritis in the animals inoculated with strain DU5814 which expressed neither haemolysin nor coagulase but did produce protein A. The expression of coagulase may not be important in the pathogenicity of *S. aureus* in this model. However, in the mutant used (DU5852) its synthesis is negatively regulated by the agr locus, whereas the secretion of protein A is enhanced. This compensation resulted in the occurrence of swollen joints even at a dose of $5 \times 10^8$ cfu of this variant. The isolation and characterisation of mutants of *S. aureus* by site-specific substitution mutation has revealed more information about the role of specific virulence factors in staphylococcal pathogenesis [16]. With a subcutaneous model of infection, strains that did not produce α-haemolysin (Hly~) produced much smaller lesions with considerable neutrophil infiltration at the inoculation site; α-haemolysin-producing strains were at least 10-fold more virulent than those which lacked the ability to produce the toxin. In the present septic arthritis model, the scoring system for clinical severity and histological damage would substantiate the importance of α-haemolysin in the virulence of *S. aureus*. As was also shown in the subcutaneous lesion assay [16], some minor differences were also apparent between protein A-positive and -negative strains. The absence of the Spa gene in some mutants did not affect the nature of the skin lesion produced, only its size. Again, the results obtained in the present study would support this conclusion.

As control of exotoxin and protein A biosynthesis is determined by the agr/hld locus within the bacterial chromosome [17], it should be possible to distinguish between the various toxins and protein A/coagulase as virulence determinants in this animal model. Most of the exotoxins are positively controlled by the agr/hld system, whereas the genes coding for protein A and coagulase are negatively controlled. Abdelnour et al. [8] showed that mice inoculated with the wild-type organism developed a higher incidence of synovitis and greater destruction of cartilage and bone than mice inoculated with agr/hld mutants. In addition, the wild-type strain survived for > 21 days within those joints that had a much higher arthritic index as compared to the agr/hld mutants. It has been suggested that *S. aureus* with an inactive agr system may have difficulty in reaching the joint cavity through the over-expression of protein A, which may interact with the host animal’s plasma proteins and lead to their sequestration within the blood vessels for easy attack by phagocytic cells. However, it has been shown in vitro [13] that such variants are more resistant to opsonophagocytosis by human neutrophils.

Patients with rheumatoid arthritis exhibit a strong chronic inflammatory response within their joints, which is probably triggered by the presence of immune complexes and pro-inflammatory cytokines. Such a pre-activated milieu may not be able to respond as readily to the appearance of *S. aureus* as the normal synovial membrane and some studies have shown that rheumatoid arthritis patients have an impaired immune response to *S. aureus* which may be responsible for their higher rates of infection by this organism. It has been proposed that this impairment is both intrinsic and extrinsic [18–20] and may involve as yet unidentified components of synovial fluid or immune complexes [19]. In this way it is possible that contact with a pathogen such as *S. aureus*, with its armoury of virulence factors, may be impossible to contain. This could explain the higher incidence of both septicemia and sequestrated infection in the joints of patients with rheumatoid arthritis.

The use of genetically engineered variants of *S. aureus* in this study has permitted the role of some of the more obvious virulence factors (protein A and α-haemolysin) to be determined in an animal model that...
more closely resembles the human disease. However, it is clear that the staphylococcus owes its pathogenicity to neither virulence factor alone and provided either protein A or α-haemolysin is produced, a significant joint infection will develop even with relatively low infective doses. It may be necessary to look for more subtle changes within the infected joint, such as the expression of specific cytokines (IL-1, IL-6, IL-8 and TNF), before the relative importance of each of these virulence factors in the development of septic arthritis can be recognised.

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References