Formylated Peptides Are Important Virulence Factors in Staphylococcus aureus Arthritis in Mice

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Background. Staphylococcus aureus is the most common pathogen causing septic arthritis in humans. The affected joints are often rapidly and permanently damaged despite antibiotic treatment, indicating that the elicited host immune response contributes substantially to joint destruction. Bacterial formylated peptides are important chemotactic molecules mediating neutrophil recruitment into infected tissues as an important first step of host defense against invading bacteria. The role of formylated peptides in S. aureus infections has been unknown.

Methods. Mice were intravenously inoculated with wild-type S. aureus strain RN4220 or its isogenic mutant strain (Δ fmt) lacking the ability to produce formylated peptides. The development of arthritis was followed clinically and histopathologically.

Results. Mice inoculated with the formyl peptide–producing wild-type strain showed a significantly increased frequency and severity of arthritis and subsequent joint destruction as compared with Δ fmt mutant strain–inoculated mice. The wild-type S. aureus strain also induced significantly more weight loss than the Δ fmt mutant strain. The recruitment of neutrophils into infected kidneys and synovial tissue was significantly higher in mice inoculated with the wild-type strain.

Conclusions. Our data show that formylated peptides function as important virulence factors in S. aureus arthritis, partly by mediating neutrophil recruitment, which contributes substantially to the joint damage.

Staphylococcus aureus is the most common pathogen in human septic arthritis, a potentially life-threatening disease that often permanently destroys the infected joints [1, 2]. Patients with underlying joint diseases, such as rheumatoid arthritis or prosthetic joint implants, and patients on immunosuppressive treatment are at increased risk for S. aureus arthritis [3, 4]. Up to half of the patients become persistently disabled due to the rapid joint destruction (erosion of cartilage and bone), which continues despite effective eradication of the bacteria with antibiotics [2]. Thus, in addition to bacterial virulence factors, the host immune response plays an important role in the joint destruction process.

This fact, together with the increasing emergence of antibiotic-resistant (in particular methicillin-resistant) S. aureus strains, implies the urgent need for development of new treatment strategies against S. aureus arthritis. Increased knowledge of S. aureus virulence factors and their interaction with the host immune response can provide a first step for development of such new therapeutic interventions.

An important early step in the immune response against invading pathogens is the chemotactic migration of phagocytes, such as neutrophils and macrophages, to sites of infection. The phagocytes are guided to the infected tissue by mediators of the host immune response as well as by gradients of bacterial products with chemotactic properties [5]. Bacteria are distinguished from eukaryotic cells by starting their protein synthesis with a formyl methionine residue at the N-terminal end, giving rise to formylated peptides [6]. It is well known that synthetic N-formyl peptides are potent neutrophil chemoattractants, and it has also been shown that N-formyl peptides are produced by S. aureus as well as by Escherichia coli and Listeria monocytogenes [7–10]. Formylated peptides from S. aureus have been shown...
to have potent chemoattractive and activating effects on mouse neutrophils in vitro and in vivo [11, 12].

However, the role of formylated peptides for virulence in S. aureus arthritis has not been studied. A powerful tool for investigating the role of putative bacterial virulence factors and their interaction with the host immune response is to study bacterial mutant strains lacking defined virulence factor genes in animal models of infection [15]. An S. aureus mutant strain lacking the formyltransferase gene necessary for production of formylated peptides was recently developed [12]. In this study we used this formyltransferase gene–deficient S. aureus strain (Afmt mutant strain) in our well-established mouse model of systemic and local S. aureus infection to investigate the importance of S. aureus formylated peptides in the development of septic arthritis in mice.

MATERIAL AND METHODS

Mice
Female NMRI mice 6–7 weeks old were obtained from B&K Universal AB and kept in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. All mice were housed in cages under standard conditions of temperature and light and were fed standard laboratory chow and water ad libitum. Permission from the local Animal Research Ethics Committee was obtained for all experiments of the study.

Bacterial Strains
The commonly used S. aureus laboratory strain RN4220 [14] and its isogenic mutant RN4220Afmt in which the gene for formyltransferase was replaced by a gentamycin-resistance gene were used [12]. The mutant strain has previously been shown not to produce formylated peptides [11].

Mouse Models of S. aureus Arthritis
Systemic septic arthritis was induced by intravenous inoculation into one of the tail veins with the wild-type S. aureus strain RN4220 (n = 40) or its isogenic Afmt mutant (n = 40). Two different doses of S. aureus (9 × 10⁶ or 1 × 10⁸ bacteria per mouse) were tested for each strain. Viability counts were performed in each separate experiment to verify the inoculated bacterial dose. All mice were regularly weighed and inspected for clinical signs of arthritis, which was defined by visible swelling and/or erythema of the joints. A clinical score (arthritic index) was determined by adding the scores for all 4 limbs after scoring each limb from 0 to 3 (0 = no swelling or erythema, 1 = mild swelling and/or erythema, 2 = moderate swelling and erythema, 3 = marked swelling and erythema). Kidneys were obtained at days 3 and 16, and blood was collected at days 12 and 16.

Local S. aureus arthritis was induced by intra-articular inoculation of the knee joints with 2 different doses (1 × 10⁷ or 1 × 10⁶ bacteria per knee) of the wild-type strain RN4220 (n = 24) or the Afmt mutant strain (n = 24). Viability counts were performed to verify the number of bacteria inoculated. Three days after intra-articular bacterial inoculation, joints were processed for histopathological evaluation as described above. In some experiments synovial tissue was cut out under an illuminated magnifier for analyses of bacterial growth and myeloperoxidase (MPO) activity.

Histopathology of Kidneys and Joints
Histopathological analysis of the joints was performed after routine fixation, decalcification, and paraffin embedding. Tissue sections from forepaws and hindpaws were stained with hematoxylin and eosin. All sections were coded before microscopic evaluation of synovitis (synovial hypertrophy/leukocyte infiltration) and joint destruction (bone-and cartilage erosion). Synovitis and destruction were scored from 0 to 3 (0 = normal appearance, 1 = mild synovitis and/or erosion of cartilage and bone, 2 = moderate synovitis and/or erosion of cartilage and bone, 3 = severe synovitis and/or erosion of cartilage and bone). A histopathological index was constructed by adding the scores from the evaluated joints in each animal. Kidneys were assessed for leukocyte infiltration after routine fixation, paraffin embedding, and hematoxylin-and-eosin staining.

Determination of Serum Interleukin-6 Protein Levels
The protein levels of interleukin-6 (IL-6) in serum were determined by a bioassay using B9 cells depending on IL-6 for their growth as described elsewhere [15].

Bacterial Growth in Kidneys and Synovial Tissue
For determination of the growth of the wild-type strain RN4220 and Afmt mutant strain, kidneys and synovial tissues were aseptically removed, homogenized, serially diluted in phosphate-buffered saline, and spread on horse blood agar plates. The number of colony forming units (CFUs) per kidney pair and synovium was determined after 24 hours of incubation at 37°C.

Measurement of MPO Activity in Synovial Tissue
Single-cell suspensions were made from the dissected synovial membranes by incubation in 1 mL of RPMI medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS, Sigma-Aldrich AB), 1 mg/mL collagenase IV (Sigma-Aldrich AB), and 0.2 mg/mL DNase (Roche) for 1 hour at 37°C. The obtained cell suspension was filtered through a nylon mesh (70 μm) and washed with RPMI medium containing 10% heat-inactivated FCS before counting the cells using an automatic cell counter (Sysmex KX-21N, Sysmex). The MPO activity was measured by lysing the cells with 20 μL lysis buffer containing 0.2% cetyltrimonium bromide (CTAB, Sigma-Aldrich AB) for 1 hour at room temperature before adding 40 μL of the peroxidase substrate 1,2-phenylenediamine dihydrochloride (OPD, Dako) diluted according to the manufacturer’s instructions and mixed with hydrogen peroxide. The absorbance was measured at 450 nm on Spectra Max 340PC.
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Figure 1. Weight change and frequency and severity of arthritis during systemic Staphylococcus aureus infection. Weight change (A) and frequency (B) and severity (C) of arthritis in mice after intravenous inoculation with Δfmt mutant or wild-type RN4220 S. aureus strain. Data in A and C are given as means ± standard error of the mean. Filled circles, Δfmt mutant strain; open circles, wild-type strain RN4220. Comparisons of groups for weight change and arthritis score were done by Mann–Whitney U test. Fisher exact test was used for comparison of arthritis frequencies. *P < .05; **P < .01; ***P < .001.

(Molecular Devices) after incubation for 1.5 hours at room temperature in the dark.

Statistical Analyses
Statistical analyses were done using GraphPad Prism software. Calculations of statistical differences between independent groups were done using the Mann–Whitney U test or Fisher exact probability test. P values < .05 were considered as statistically significant.

RESULTS
Clinical Course of S. aureus Arthritis Is Aggravated by Formylated Peptides
The role of formylated peptides in the development of systemic S. aureus septic arthritis was studied by inoculating mice intravenously with 1 × 10^8 CFU per mouse of the wild-type strain RN4220 or its isogenic Δfmt mutant strain lacking the ability to produce formylated peptides. Mice inoculated with the wild-type strain had already lost significantly more weight 3 days after bacterial inoculation, compared with mice inoculated with the Δfmt mutant strain (Figure 1A). Both the severity and frequency of clinical arthritis was increased after inoculation with wild-type strain, as all 9 mice inoculated with the wild-type strain had developed arthritis at day 16, whereas none of the mice inoculated with the Δfmt mutant strain had signs of arthritis (P < .001, Figure 1B and 1C).

There was a trend toward higher IL-6 protein levels in serum at day 16 in response to intravenous inoculation of the wild-type strain (median, 253 [range, 60–390 pg/mL]) compared with the levels in response to intravenous inoculation of the Δfmt mutant strain (median, 106 [range 30–330 pg/mL]).

Statistical Analyses were done using GraphPad Prism software. Calculations of statistical differences between independent groups were done using the Mann–Whitney U test or Fisher exact probability test. P values < .05 were considered as statistically significant.

The results were similar when a lower bacterial dose (9 × 10^6 CFU/mouse) was used. The frequency of arthritis was 56% (5 of 9) in mice inoculated with wild-type strain compared with 20% (2 of 10) in mice inoculated with the Δfmt mutant strain. The median arthritis scores were 1 (range, 0–3) and 0 (range, 0–1), for wild-type and Δfmt mutant strains, respectively (data not shown). Regardless of the bacterial dose, the mortality was low; one mouse died after inoculation with the wild-type strain, whereas none of the mice inoculated with the Δfmt mutant strain died.

S. aureus Formylated Peptides Worsen Synovitis and Joint Destruction
Histopathological evaluation of joints 16 days after intravenous S. aureus inoculation revealed significantly more severe synovitis and joint destruction in mice inoculated with the wild-type strain compared with mice inoculated with the Δfmt mutant strain (Figure 2A and 2B).

Similar findings were obtained using the lower bacterial dose; inoculation with the wild-type strain significantly increased the erosivity of arthritis with a median score of 1.0 (range, 0–3) whereas inoculation of the Δfmt mutant strain did not lead to any destruction at all (P = .028). The median synovitis score was 2.0 (range, 0–6) after inoculation with the wild-type strain compared with 0.5 (range, 0–2) after inoculation with the Δfmt mutant strain (data not shown).

Impact of S. aureus Formylated Peptides on Bacterial Load
The Δfmt mutant strain has been shown to have impaired growth in vitro [12]. Because of this finding, we decided to investigate whether the lower inflammatory response and milder joint destruction seen in mice inoculated with this particular bacterial strain could be explained by a lower bacterial load in these mice. In line with the in vitro data, a significantly decreased bacterial load was seen early in the infection (day 3) in mice inoculated with the Δfmt mutant strain. However, this difference had disappeared at a later time point (day 16; Figure 3A). Furthermore, at day 3 after intra-articular injection of bacteria into the knee joints, no differences in bacterial growth were seen in synovial tissue (Figure 3B).

S. aureus Formylated Peptides Contribute to Neutrophil Recruitment In Vivo
We next investigated whether the increased inflammatory response and joint damage could be due to an increased
recruitment of neutrophils induced by the formylated peptide–producing wild-type strain. Histopathological examination of the kidneys 3 days after intravenous inoculation of *S. aureus* revealed an influx of neutrophils, found in multifocal inflammatory lesions scattered in the renal cortex and pelvis, in 22 of 25 mice (88%) inoculated with the wild-type strain, while only 9 of 25 (36%) of the mice inoculated with the Δfmtn mutant strain had infiltrating neutrophils in their kidneys (*P* = .0003, Figure 4A and 4D). However, this difference in neutrophil infiltration could depend on the slower growth of the Δfmtn mutant strain. Therefore, we inoculated 15 mice per group with the same doses of the Δfmtn mutant strain and the wild-type strain.

Figure 2. Histopathological evaluation of arthritis in systemic *Staphylococcus aureus* arthritis. Histopathological scoring of synovitis and erosion of cartilage and bone at day 16 after intravenous inoculation with Δfmtn mutant or wild-type *S. aureus* strain (A). Photomicrographs (B) of hematoxylin and eosin–stained tissue sections of knee joints from mice inoculated with Δfmtn mutant strain (left panel) and wild-type strain (right panel). Original magnification × 50. Filled circles, Δfmtn mutant strain; open circles, wild-type strain RN4220. Bars represent medians. Comparisons of groups for synovitis and erosivity were done by Mann–Whitney *U* test. Abbreviations: E, erosion; S, synovitis. **P < .01; ***P < .001.

Figure 3. Bacterial growth in kidneys and synovial tissues. Number of colony forming units (CFUs) in kidneys at day 3 and day 16 after intravenous inoculation (A) and in joints 3 days after intra-articular inoculation (B) with Δfmtn mutant or wild-type RN4220 *Staphylococcus aureus* strain. Filled circles, Δfmtn mutant strain; open circles, wild-type strain RN4220. Bars represent medians. Mann–Whitney *U* test was used for comparison between groups. *P* < .05.
Figure 4. Neutrophil infiltration into *Staphylococcus aureus*-infected tissues. Frequency of neutrophil infiltration in tissue sections after intravenous (kidneys) or intra-articular (synovia) inoculation of mice with Δfmt mutant (filled bars) or wild-type *S. aureus* strain (open bars, A). Number
respectively, and then performed viability counts at day 3 on half of each kidney from each mouse. Six mice from each group were found to harbor very similar amounts of bacteria in their kidneys (Figure 4B). The remaining halves of the kidneys from these 6 mice were then analyzed for neutrophil infiltration; mice infected with the wild-type strain had significantly more neutrophils than mice carrying comparable numbers of the \( \text{Afmt} \) mutant strain (\( P = .026, \) Figure 4C).

The wild-type strain also more frequently induced influx of neutrophils into the joints after intra-articular inoculation with \( 1 \times 10^5 \) bacteria per joint; 8 of 10 mice had infiltrating neutrophils in their joints compared with only 2 of 10 mice inoculated with \( \text{Afmt} \) mutant strain (\( P = .023, \) Figure 4A and 4E). This finding corresponded well with the more severe synovitis seen in mice 3 days after intra-articular inoculation of the wild-type strain than after inoculation with the \( \text{Afmt} \) mutant strain (\( P = .0499, \) not shown). Similar results were obtained using a higher bacterial dose (\( 1 \times 10^6 \) per joint); 5 of 8 mice inoculated with the wild-type strain had infiltrating neutrophils in their joints as compared with 1 of 8 mice inoculated with \( \text{Afmt} \) mutant strain. There were no differences in myeloperoxidase activity in synovial tissue obtained from wild-type–inoculated mice (median optical density [OD], 0.31 [range, 0.16–0.92]) and \( \text{Afmt} \) mutant–inoculated mice (median OD, 0.21 [range, 0.12–0.37]).

**DISCUSSION**

Even though it has been >30 years since Schiffman et al [7] demonstrated that synthetic formylated peptides are important for neutrophil chemotaxis in vitro, their role during infections in vivo remains largely unknown. In the present study we investigated the role of formylated peptides for the development of \( S. \) aureus arthritis by inoculating mice with a wild-type formyl peptide–producing \( S. \) aureus strain or its isogenic \( \text{Afmt} \) mutant strain, which is unable to produce formylated peptides. We found that the ability of \( S. \) aureus to produce formylated peptides played a pivotal role for the development of \( S. \) aureus arthritis. Mice inoculated with the formyl peptide–producing \( S. \) aureus wild-type strain showed a higher frequency and severity of arthritis as well as more destructive joint disease than mice inoculated with the isogenic \( \text{Afmt} \) mutant strain. The joint destruction was accompanied by an increased infiltration of neutrophils into the joints of the mice inoculated with the formyl peptide–producing \( S. \) aureus strain. Importantly, the increased joint inflammation and destruction after inoculation with the formyl peptide–producing strain was not explained by a long-term impaired growth rate of the \( \text{Afmt} \) mutant strain.

\( S. \) aureus and \( E. \) coli have both been shown to release formylated peptides into the supernatants when cultured in vitro [9, 10]. However, it has been hitherto unknown whether bacteria also release formylated peptides during in vivo infections. Our findings of an increased inflammatory response, as shown by increased weight loss, worsened synovitis, and local neutrophil accumulation in the infected tissues (ie, kidneys and synovia) in mice inoculated with the formyl peptide–producing wild-type strain, compared with the \( \text{Afmt} \) mutant strain, suggest that formylated peptides indeed are released from \( S. \) aureus in vivo during infection.

The role of neutrophils in \( S. \) aureus infections is dual: on one hand they are needed for bacterial clearance and host survival, and on the other hand, they contribute significantly to tissue damage by their release of bacteria- and tissue-degrading enzymes and free radicals. It has been shown that deletion of neutrophils leads to decreased bacterial clearance and higher mortality and worsens arthritis early in \( S. \) aureus sepsis and septic arthritis [16]. In the present study we found that inoculation of the \( \text{Afmt} \) mutant strain was associated with decreased neutrophil infiltration and less severe joint damage, despite similar bacterial loads. It might be that the few infiltrating neutrophils we found in the tissues of mice inoculated with the \( \text{Afmt} \) mutant strain were sufficient to prevent overwhelming bacterial growth, thus preventing tissue damage induced by bacterial molecules. The finding that infiltrating neutrophils were also produced in the \( \text{Afmt} \) mutant strain–inoculated mice is in good agreement with the reported presence of other \( S. \) aureus molecules with neutrophil chemoattractive properties in culture supernatants of the mutant [11]. In addition, it has been shown that virulence of MRSA and other highly pathogenic \( S. \) aureus strains depend on their production of phenol-soluble modulin peptide toxins, which can attract neutrophils through binding to the human formyl peptide receptor 2 [17, 18].

Disruption of the \( \text{fmt} \) gene in \( S. \) aureus as well as in \( E. \) coli has earlier been shown to impair bacterial growth in vitro [11, 19]. Thus, the increased virulence of the wild-type strain observed in our study could only have been due to higher bacterial persistence in mice inoculated with this strain. However, even though
we found higher bacterial numbers in the kidneys of wild-type–
inoculated mice early in the infection (at day 3), we did not see any differences regarding the bacterial density in kidneys at a later time point (day 16), when the differences in severity of arthritis were even larger than early on. In addition, there were no differences in the bacterial load early after intra-articular inoculation. This is in contrast to the findings by Margolis et al [12], who found lower recovery of their fmt mutant using a murine abscess model mimicking foreign body infection. This discrepancy between our findings and the findings of Margolis et al might be explained by the different inoculation routes of S. aureus or by the fact that different fmt mutant strains from different parent strains were used.

Instead of depending on impaired growth, we propose that the absence of formylated peptides in our fmt mutant strain leads to a weaker recognition by the host immune response, thereby not causing such severe inflammation of the joints.

Even though the N-terminal formylated methionine residues often are removed from the bacterial proteins through the action of peptide deformylase, many proteins keep their N-terminal formylated methionine molecule [20]. Thus, another possible explanation for the milder arthritis seen in the formyl transferase mutant strain–inoculated mice is the possible dependence of other S. aureus virulence factors on the presence of an N-formyl group, and thereby on a functional fmt gene, for their expression and function. This needs to be further investigated.

In conclusion, our study shows that formylated peptides play an important role in S. aureus arthritis and that this might, at least partly, be dependent on the neutrophil chemoattractive effects of staphylococcal formylated peptides. Thus, inhibition of formyl peptide signaling, in combination with antibiotics, might be a future treatment strategy to prevent joint destruction in S. aureus arthritis.

Notes

Acknowledgments. We thank Margareta Verdrengh and Berit Ertman-Ericsson for excellent technical assistance and Peter S. Margolis for providing the fmt mutant strain.

Funding. This work was supported by grants from the Swedish Research Council (grant number 521-2007 to A. T.), the Professor Nanna Svartz Foundation (grant numbers GLS-98561 and GLS-42751 to C. L.), Reumatikerförbundet i Göteborg (to I. M. J.), the Swedish Rheumatism Association (to I. M. J.), Amlöv’s Foundation (to C. L.), Thölen and Kristler Foundation (to I. M. J.), Golje Memory Foundation (to I. M. J.), and the German Research Council (grant number SFB685 to A. P.).

Potential conflicts of interest. All authors: No reported conflicts.

References