Research article
Staphylococcal peptidoglycans induce arthritis

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Abstract

Staphylococcus aureus is one of the most important pathogens in septic arthritis. To analyse the arthritogenic properties of staphylococcal peptidoglycan (PGN), highly purified PGN from *S. aureus* was intra-articularly injected into murine joints. The results demonstrate that PGN will trigger arthritis in a dose-dependent manner. A single injection of this compound leads to massive infiltration of predominantly macrophages and polymorphonuclear cells with occasional signs of cartilage and/or bone destruction, lasting for at least 14 days. Further studies showed that this condition is mediated by the combined impact of acquired and innate immune systems. Our results indicate that PGN exerts a central role in joint inflammation triggered by *S. aureus*.

Keywords: arthritis, peptidoglycan, *Staphylococcus aureus*

Introduction

Bacterial arthritis is the most rapidly progressive destructive joint disease in man. Clinical symptoms of septic arthritis include red, swollen, warm, painful and dysfunctional joints. Other destructive joint diseases, including rheumatoid arthritis, are connected to an increased incidence of bacterial arthritis. Indeed, the yearly incidence of arthritis in the general population is 0.002–0.01% but 0.03–0.07% in patients with rheumatoid arthritis, and mortality continues to be high (10–16%) [1].

Staphylococcus aureus is the most common bacterium that causes septic arthritis. Staphylococci produce a large number of extracellular and cell-associated molecules that may contribute to virulence [2–4]. These factors enable the bacteria to evade host defenses, and thus to establish infection, but they may also trigger a cascade of host proinflammatory and immunomodulating molecules [2].

Peptidoglycan (PGN) is the major component of the cell wall of Gram-positive bacteria and is composed of long sugar chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues, which are highly cross-linked via peptide side chains. The peptide side chain consists of alternating L- and D-amino acids, up to four or five in length, and is connected to the COOH group of N-acetylmuramic acid. Among different bacterial species, the structure of the sugar chains is highly conserved, while the composition of the peptide subunits varies [5]. Some recent reports indicate that staphylococcal PGN and lipoteichoic acid (LTA) activate leukocytes, stimulate the generation of proinflammatory cytokines, and hence, cause an inflammatory response [6–8]. Indeed, PGN and LTA are essential in the induction of nitric oxide synthase, shock, and multiple organ failure [9].

To investigate the possible role of staphylococcal PGN during septic arthritis, we have intra-articularly administered

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IL = interleukin; LTA = lipoteichoic acid; mAb = monoclonal antibody; NK = natural killer; PBS = phosphate buffered saline; PGN = peptidoglycan; TNF = tumour necrosis factor.

PGN from *S. aureus* into murine knee joints. Our results show that staphylococcal PGN induces arthritis. In addition we show that this condition is mediated by concerted action of macrophages and acquired immunity.

Materials and methods
Mice and reagents
BALB/c, C57BL/6, and NMRI mice were purchased from ALAB (Stockholm Sweden). SCID mice and their congenic strain CB17 were purchased from M&B (Bomholtvej, Denmark). All mice were housed in the animal facility of the Department of Rheumatology (University of Göteborg, Sweden). Male mice, six to eight weeks old, were used in all experiments. The hybridoma cells secreting Rb6-8C5 were a kind gift from Dr R Coffman (DNAX Research Institute, Palo, CA, USA). Hybridoma PK136 was obtained from Dr Sjögren-Jansson (Wallenberg Laboratory, Göteborg, Sweden). Etoposide was purchased from Bristol-Myers Squib (Bromma, Sweden). The PGN and fragments of PGN were prepared as previously described [9,10]. LTA was obtained from Sigma Chemical Co (St Louis, MO, USA).

Injection protocol
PGN dissolved in phosphate buffered saline (PBS) in a volume of 10 μl was injected intra-articularly in the knee joints of different mice. The contralateral knee joint was always used as a negative control, and was injected with same volume of PBS. All the intra-articular injections were performed on anesthetized mice approaching from the frontal aspect of the knee joint, just below the patella.

Histopathological examination
After routine fixation, decalcification, and paraffin embedding, tissue sections from knee joints were cut and stained with hematoxylin and eosin [3]. All of the slides were coded and assessed in a blinded manner. The specimens were evaluated with regard to synovial hypertrophy, pannus formation, and cartilage-subchondral bone destruction. The extent of synovitis was judged on an arbitrary scale, from grade 0 (no signs of inflammation) and grade 1 (mild inflammation characterized by hyperplasia of the synovial lining layer), to grades 2 and 3 (increasing degree of extended inflammation characterized by influx of inflammatory cells scattered throughout the synovial tissue).

Immunohistochemical examination
The knee joints were removed and demineralized by enzymatic procedures detailed previously [3]. The demineralized specimens were mounted on cryostat chucks, frozen in isopentane that had been prechilled in liquid nitrogen, and kept at −70°C until cryosectioned. Serial cryosection 6 μm thick were stained with a monoclonal rat antibody to mouse CD11b (Mac-1), CD4 (GK1.5), CD8 (53.6.7) followed by incubation with biotinylated secondary antibodies, avidin–biotin–peroxidase complexes, and 3-amino-9-ethyl-carbazole containing H_2O_2. All sections were counterstained with Mayer´s hematoxylin [3].

Monocyte depletion
Etoposide is a cytotoxic drug known to selectively deplete the monocyte population [11]. C57B1/6 mice were subcutaneously injected with 12.5 mg/kg body weight of etoposide on 3 consecutive days before intra-articular injection with PGN and on either 2 or 5 consecutive days after it. Control mice received the same volume of the vehicle. Flow cytometry analysis revealed that peripheral blood monocytes decreased significantly as compared with the numbers in controls (10.5×10^4 ml versus 51.7×10^4 ml, $P < 0.01$).

Neutrophil depletion
The mAb RB6-8C5 is a rat IgG2b that selectively binds to and depletes mature mouse neutrophils and eosinophils [12]. BALB/c mice were injected intraperitoneally with 1 mg of either mAb RB6-8C5 or the IgG rat anti-ovalbumin mAb as a control, 2 hours prior to intra-articular injection with PGN. This procedure depleted neutrophils by 90% as previously reported [12].

Natural killer-cell depletion
Natural killer (NK)-cell depletion was performed by intraperitoneal inoculation with 200 μg PK136 mAb, 1 day prior to and 2 days after intra-articular injection of PGN in C57B1/6 mice. The O1C5B2 mAb was used as control [4,13]. This procedure has been shown to reduce the number of NK cells by 90% [13].

Statistical analyses
The differences between mean values were tested for significance with the Student's two-tailed *t*–test. $P < 0.05$ was considered as significant.

Results
Induction of arthritis by *S. aureus* peptidoglycan
S. aureus PGN was directly injected into murine knee joints. Within one day, joints were found to be macroscopically swollen and the swelling lasted for 10 days after intra-articular inoculation of 100 μg of PGN.

Histopathological analysis revealed a very strong synovial inflammation (Fig. 1b) and frequent occurrence of granulomas when arthritis was induced by 100 μg of PGN. There was synovial hypertrophy and occurrence of infiltrating cells in the synovial lining cell layer, deep in the sublining space, as well as in surrounding synovial vessels in arthritic joint sections. Granulomas were found in the majority of samples, and in a few samples (6 of 18) pannus formation, cartilage and/or bone destruction was visible. Arthritis triggered by 100 μg PGN was visible within 2 hours after the injection and lasted for at least 2 weeks. The maximal frequency and severity of arthritis were noted on day 3 after the injection (Fig. 2).

Figure 1
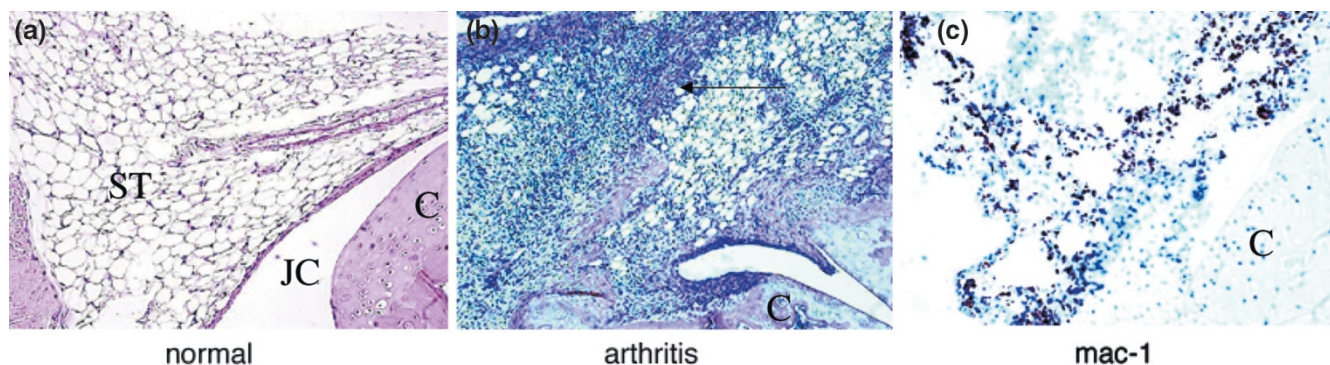
Photomicrographs showing the histopathologic and immunohistochemical features of arthritis induced by peptidoglycan. **(a)** Normal histopathologic appearance of a mouse knee joint following injection with phosphate-buffered saline. **(b)** Histopathology of an arthritic knee joint of a mouse 3 days after intra-articular inoculation with peptidoglycan, showing infiltration of inflammatory cells in synovial tissue and granulomas within the synovium. **(c)** Immunohistochemistry of an arthritic knee joint, showing synovial expansion of Mac-1-expressing cells three days after intra-articular inoculation with peptidoglycan. JC, joint cavity; C, cartilage; ST, synovial tissue.

To assess the optimal amount of PGN for triggering arthritis, different doses of PGN (0, 1, 10, 25, 50, 75, 100 µg/knee) were used in one experiment. Fifty percent of mice developed arthritis after injection of 1 and 10 µg. Severity of arthritis is 0.5 ± 0.58, 0.75 ± 0.96, respectively. The dose of 25 µg or more gave rise to arthritis in all the knee joints analyzed.

We examined 6 different mouse strains, including Swiss, BALB/c, C57B1/6, C3H/HeN, C3H/HeJ and CB17 to assess their susceptibility to arthritis. We found that arthritis was induced by PGN in all of these strains.

Immunohistochemical features of arthritic joints
The majority of the lining cells in the nonproliferative synovial tissue was stained with Mac-1, recognizing resident macrophages. T lymphocytes were not observed in healthy synovium. In sections of arthritic joints, a large population of synovial cells was stained with Mac-1 (Fig. 1c). These cells, which have a morphology of macrophage (i.e. mononuclear cells), were found both within the thickened synovial lining layer and within the deeper synovial tissue, The predominance of Mac-1$^+$ cells was evident at all stages of inflammation. A few CD4$^+$ and CD8$^+$ T cells were also found.

The role of immune cells in the development of arthritis triggered by peptidoglycan
Immunohistochemical results from joints injected with PGN demonstrated that there was an abundance of Mac-1$^+$ cells and few T lymphocytes at all stages of arthritis. These data suggest that Mac-1$^+$ cells and T lymphocytes might play a role in the development of PGN-triggered arthritis.

Figure 2
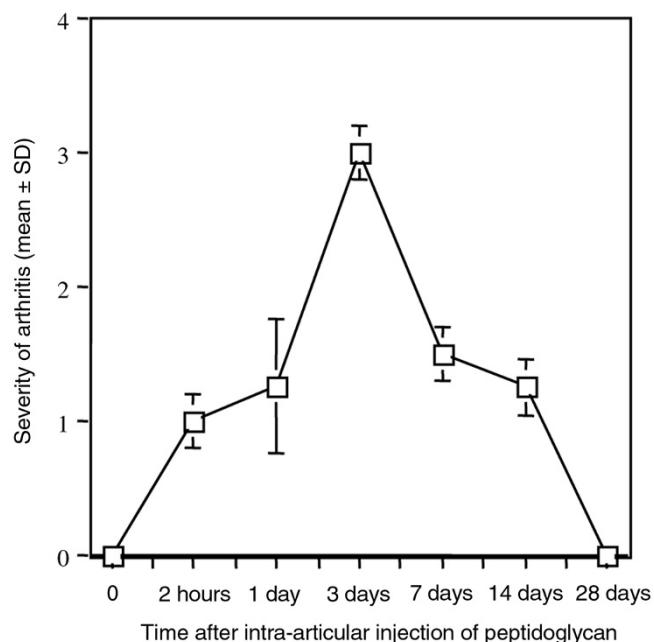
Kinetics of arthritis induced by peptidoglycan ($n = 4$ per group) in NMRI mice. Values are shown as mean $\pm$ SD.

We used etoposide to deplete the monocyte population and thereby assess the role of tissue infiltrating macrophages. Despite 80% depletion of monocytes, however, the incidence and severity of arthritis were not different between monocyte-depleted and control mice (Table 1).

Table 1

The frequency and severity of arthritis triggered by intra-articular injection of 100 µg of peptidoglycan in mice depleted of neutrophils, NK cells, or monocytes

Group	Mice	Incidence	Severity
RB6-8C5	BALB/c	10/10	2.0 ± 0.7
Control	BALB/c	9/9	2.3 ± 0.8
PK136	C57BL/6	10/10	1.8 ± 0.8
Control	C57BL/6	10/10	2.2 ± 0.6
Etoposide	C57BL/6	8/8	1.6 ± 0.7
Control	C57BL/6	10/10	2.1 ± 0.5
SCID	CB17	9/9	1.6 ± 0.7
Control	CB17	10/10	2.7 ± 0.5

RB6-8C5 is an IgG2b antibody that depletes neutrophils in BALB/c mice. Etoposide is a cytotoxic drug that selectively depletes monocytes. PK136 is an IgG2a antibody that depletes natural killer cells. SCID mice lack T and B cells.

To assess the role of neutrophils, we used RB-8C5 antibody to deplete these cells. Again neutrophil-depleted mice did not differ from control mice in their development of arthritis mediated by PGN (Table 1). We next determined whether NK cells exert any role in arthritis triggered by PGN. We depleted NK cells using PK136 antibody, and as shown in Table 1, the incidence and severity of arthritis were not different between NK-cell-depleted mice and the control mice. We also assessed the role of T and B cells using SCID mice, which lack T and B cells but have an intact population of macrophages and NK cells. Histopathologic results from SCID mice and their congenic littermates (CB17) demonstrated that the severity and incidence of arthritis were not significantly different between the groups (Table 1).

Lastly, to evaluate the role of synergism of immune cells in the development of arthritis mediated by PGN, the following experiment using SCID mice was performed. RB6-8C5 antibody and etoposide were used to deplete neutrophils and macrophages in SCID and BALB/c mice. The results demonstrate that the incidence and severity of arthritis mediated by PGN in monocyte-depleted and neutrophil-depleted SCID mice were significantly reduced ($P < 0.05$) (Table 2). When neutrophils alone were depleted in SCID mice, the incidence and severity of arthritis mediated by PGN did not show a clear difference compared with the control group. In contrast, macrophage alone depletion in SCID mice resulted in decreased incidence and severity of arthritis mediated by PGN compared with control group. These results provide strong evidence for the synergistic role of monocyte/macrophage and T/B lymphocytes in initiating and maintaining arthritis triggered by PGN.

Table 2

The incidence and severity of arthritis after intra-articular injection of 100 µg peptidoglycan to SCID mice depleted of neutrophils and monocytes

| Group	Number of mice	Incidence (%)	Severity
CB17	10	100	2.8 ± 0.4
SCID + RB6-8C5	10	100	1.6 ± 0.5
SCID + etoposide	8	50	0.5 ± 0.5
SCID+ RB6-8C5 + etoposide	8	50	0.6 ± 0.7
BALB/c+RB6-8C5 + etoposide	10	70	1.5 ± 1.1

Digested peptidoglycan does not induce arthritis

To further elucidate the structural elements of PGN that are essential to induce arthritis, PGN (9 mg/ml) from *S. aureus* was digested for 18 hours with lysostaphin (150 µg/ml) or cellosyl (150 µg/ml) before intra-articular injection into knee-joints. Lysostaphin was used to hydrolyse the glycine crossbridge of the PGN, resulting in long chains of soluble material [9]. Cellosyl is a muramidase that hydrolyses the bond between N-acetylglucosamine and N-acetylmuramic acid, producing small soluble muropeptides (some of which are crosslinked) [10]. We used these enzymes to digest PGN into different fragments and analysed whether these fragments are responsible for the inflammatory response. Our results, however, showed that the fragments from PGN digested by either lysostaphin or cellosyl did not give rise to any inflammatory response in joints (Fig. 3).

Discussion

The purpose of this work was to investigate the role of PGN from *S. aureus* in the induction of arthritis. Our results reveal that it induces joint inflammation, and it may, therefore, play an important role in septic arthritis.

The synergistic effect of macrophages and T cells is essential to trigger arthritis. There are several lines of support for this suggestion. Arthritis occurs within 2 hours after administration of PGN, rendering it highly improbable that immune responses mediated by lymphocytes alone would trigger such a process without prior exposure. This rapid onset of arthritis indicates that innate immune responses are involved at this stage of disease. Also, our experiment demonstrated that absence of either neutrophils, NK cells, monocytes, or T/B cells does not significantly affect the development of arthritis induced by PGN. In contrast, depletion of circulating monocytes in SCID mice decreased by half the prevalence of arthritis. These findings are further supported by immunohistochemical analy-

Figure 3
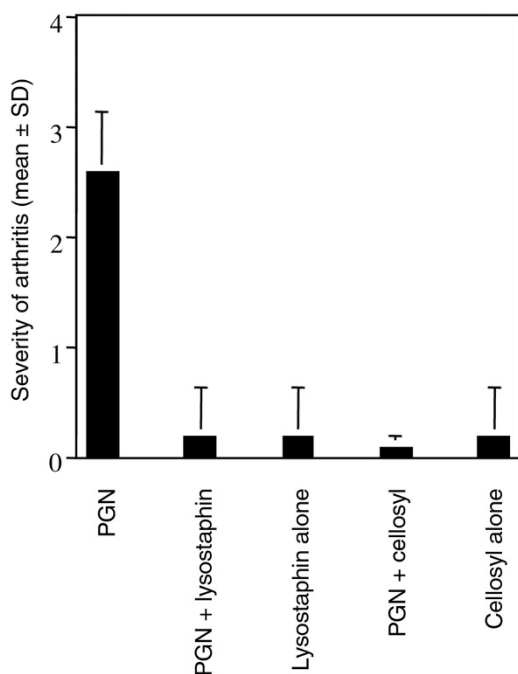
Severity of arthritis in NMRI mice after intra-articular inoculation with intact and digested peptidoglycan. The knee joints were inoculated with peptidoglycan (PGN) (100 µg/joint), PGN digested by lysostaphin (180 µg/joint), lysostaphin alone (150 µg/joint), PGN digested by cellosyl (180 µg/joint), or cellosyl alone (150 µg/joint) ($n = 5$ per group). All mice were killed three days after the inoculation and histopathological examination was performed. Values are shown as mean ± SD.

sis showing that PGN-triggered arthritis was characterised by an influx of mononuclear Mac-1$^+$ and CD4$^+$ cells.

Several studies have shown that staphylococcal PGN stimulates human monocytes to release tumour necrosis factor-alpha (TNF-α), IL-1, and IL-6 [7,8,14], and that T cells producing TNF-α and IL-1 in response to stimulation with Gram-positive bacterial cell wall components [15] are known to exert proinflammatory activities in septic [16], and aseptic arthritis [17,18], being able to mediate cartilage and bone destruction. Indeed, it has been demonstrated that a single intra-articular injection of TNF-α or IL-1 induces acute synovitis [19,20], whereas neutralisation of TNF-α decreases the severity of the inflammation and joint destruction in collagen-II-induced-arthritis [2,22].

Surprisingly, in this study we found that fragments from PGN digested with lysostaphin or cellosyl do not induce arthritis. Previous studies demonstrated that the endopeptidase lysostaphin significantly enhanced the nitrite formation [9]. Interestingly, these results indicate that there are clearly different mechanisms between PGN-induced local inflammatory response and PGN-induced systemic inflammatory response.

Our results demonstrate that PGN induces arthritis and suggest an important pathogenic role for this molecule in septic arthritis. Moreover, in the cases of reactive arthritis and rheumatoid arthritis, the role of PGN as a perpetuating stimulus for a local inflammatory response in the joints should be evaluated. Our findings may have implications for the treatment of bacterial arthritides. Eradication of bacteria using antibiotics might not be sufficient to eliminate disease symptoms, since the proinflammatory bacterial products including PGN released from resident bacteria in concert with bacterial DNA, might promote a continuous inflammatory response. Indeed, previous epidemiological studies have indicated that despite antibiotic treatment, and therefore eradication of all live bacteria, inflammation and joint destruction continue [1]. Some clinical studies suggest in fact that irrigation of joints might be beneficial in cases of septic arthritis [23]. One potential effect of such irrigation would be to decrease the local levels of PGN. Further treatment strategies should include attempts to minimize bacterial growth, together with blockade of the proinflammatory effect of PGN.

Conclusion

Our results indicate that *S. aureus* PGN exerts a central role in joint inflammation triggered by *S. aureus* and this condition is mediated by the combined impact of the acquired (T and B cells) and the innate (monocytes/macrophages) immune systems.

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