**Streptococcus pyogenes** Infection Induces Septic Arthritis with Increased Production of the Receptor Activator of the NF-κB Ligand

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Bacterial arthritis is a rapidly progressive and highly destructive joint disease in humans, with *Staphylococcus aureus* and *Neisseria gonorrhoeae* the major causative agents, although beta-hemolytic streptococci as well often induce the disease. We demonstrate here that intravenous inoculation of CD-1 mice with the group A streptococcus (GAS) species *Streptococcus pyogenes* resulted in a high incidence of septic arthritis. Signs of arthritis emerged within the first few days after infection, and bacterial examinations revealed that colonization of the inoculated GAS in the arthritic joints persisted for 21 days. Induction of persistent septic arthritis was dependent on the number of microorganisms inoculated. Immunohistochemical staining of GAS with anti-GAS antibodies revealed colonization in the joints of infected mice. Cytokine levels were quantified in the joints and sera of infected mice by using an enzyme-linked immunosorbent assay. High levels of interleukin-1β (IL-1β) and IL-6 were detected in the joints from 3 to 20 days after infection. We noted that an increase in the amount of receptor activator of NF-κB ligand (RANKL), which is a key cytokine in osteoclastogenesis, was also evident in the joints of the infected mice. RANKL was not detected in sera, indicating local production of RANKL in the infected joints. Blocking of RANKL by osteoprotegerin, a decoy receptor of RANKL, prevented bone destruction in the infected joints. These results suggest that GAS can colonize in the joints and induce bacterial arthritis. Local RANKL production in the infected joints may be involved in bone destruction.

The group A streptococcus (GAS) *Streptococcus pyogenes* is a gram-positive bacterial pathogen that causes infections such as pharyngitis, bacteremia, and necrotizing fasciitis, as well as postinfectious sequelae, such as acute rheumatic fever (7). Further streptococcal infections can induce acute rheumatic fever and poststreptococcal reactive arthritis (3, 20, 27). This type of reactive arthritis has been suggested to be due to the production of antibodies to the infecting agent, which cross-react with joint synovial tissue or cartilage (3). In addition to reactive arthritis, infectious septic arthritis has been reported as a clinical manifestation of GAS infection (10, 27).

Bacterial infections can be localized in joints, causing septic arthritis, the most rapidly progressing joint disease, after which permanent joint damage develops in most cases, and mortality continues to be high (10). *Staphylococcus aureus* and *Neisseria gonorrhoeae* are the most frequently isolated bacterial species associated with septic arthritis; however, GAS is the next most common bacterium isolated from septic joints of patients and accounts for 8 to 16% of cases of septic arthritis (10, 27). Therefore, GAS induces several types of arthritis, including poststreptococcal reactive arthritis, rheumatic fever, and septic arthritis.

To study the pathogenesis of septic arthritis, a murine model of the disease has been developed using *S. aureus* (5) and the group B streptococcus (GBS) *Streptococcus agalactiae* (24, 34). Certain bacterial virulence factors such as superantigens and exotoxins have been implicated in *S. aureus* arthritis (33), and host immune responses were reported to be important in the induction and progression of this disease (12, 32, 35). Inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) participate in the pathogenesis of septic arthritis (4). Regarding GAS, it has been established that injection of cell wall or peptidoglycan-polysaccharide fractions from the organism produces acute joint lesions in rats and mice (6, 15), and such streptococcal cell wall arthritis has been used as a model of rheumatoid arthritis. However, investigations of septic arthritis induced by *S. aureus* or GBS have revealed that septic arthritis is not induced by inactivated cells or sonicated cellular extracts containing bacterial peptidoglycan, polysaccharides, and proteins (5, 34); thus, the relationship between septic arthritis and streptococcal cell wall arthritis is not well known.

Pathogenic bone destruction is associated with the up-regulation of osteoclastic bone resorption. Until recently, the molecular mechanisms of osteoclast differentiation and up-regulation of bone resorption have been poorly understood. However, recent investigations have identified members of the TNF family of ligands and their receptors as critical regulators of osteoclastogenesis. The cytokine receptor activator of NF-κB ligand (RANKL) is now recognized as a critical cytokine in osteoclast differentiation (2, 11, 30). RANKL induces osteoclast differentiation from osteoclast precursor cells in the presence of macrophage colony-stimulating factor (11, 30), and RANKL-deficient mice show severe osteopetrosis, revealing the importance of the cytokine in osteoclastogenesis (8, 17). Although recent reports have suggested the involvement of

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RANKL in rheumatoid arthritis (18, 25), its role in septic arthritis has not been studied.

Osteoclastogenesis is blocked in the presence of osteoprotegerin (OPG) (11, 28, 30, 37). OPG is a soluble decoy receptor that inhibits osteoclast formation, function, and survival by preventing the binding of RANKL to its receptor, which is present on osteoclast precursors and mature osteoclasts (11, 30, 37). Mice with null mutations of the OPG gene exhibit severe osteoporosis (28), while mice with ablated RANKL genes exhibit osteosclerosis (11, 17, 30).

In the present study, we found that systemic challenge of mice with GAS resulted in chronic infection with a high incidence of septic arthritis. High levels of proinflammatory cytokines including RANKL were detected in the arthritic joints of the infected mice. In addition, administration of OPG inhibited the bone destruction induced by GAS infection.

MATERIALS AND METHODS

Mice and reagents. Female outbred CD-1 mice, 6 weeks old, were obtained from Charles River Japan (Yokohama, Japan) and used throughout the experiments. Recombinant mouse OPG (a recombinant OPG fused to human immunoglobulin G [IgG] Fc) was purchased from R&D Systems (Minneapolis, Minn.).

Administration of bacteria. This study was reviewed and approved by the Animal Care Committee of Osaka University. GAS strain JRS4 (serotype M6; streptomycin resistant) was provided by E. Hanski (The Hebrew University Hadassah Medical School, Jerusalem, Israel) (13). Strains SSI-1 (M3) and SSI-9 (M1) were clinical isolates from patients with toxic shock-like syndrome as described previously (14, 21). The microorganisms were grown at 37°C in Todd-Hewitt broth medium (BD Bioscience, Sparks, Md.) supplemented with 0.2% yeast extract (BD Bioscience) (THY) until the mid-log phase. The bacterial cells were washed and diluted in serum-free Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo.). The GAS inoculum size was determined turbidimetrically at 600 nm, and the number of live bacterial cells was confirmed by enumeration of the CFU on THY agar plates. On day 0, the desired number of GAS organisms was inoculated intravenously (i.v.) via a tail vein in a 0.5-ml volume of DMEM. Control mice were injected in the same way with 0.5 ml of DMEM. In order to demonstrate the involvement of RANKL in bone destruction induced by the GAS infection, recombinant OPG (1 mg/kg of body weight/day) was administered subcutaneously daily from day 3 to day 9.

Clinical evaluation of arthritis. Mice infected with GAS strain JRS4 were examined 1 day after infection and then every other day for 3 weeks to evaluate the clinical features of the disease—in particular, the presence of joint arthritis. The degree of erythema or swelling was evaluated for each hind paw and recorded as follows: 0 points, no swelling or erythema; 1 point, mild erythema; 2 points, mild swelling and moderate erythema; and 3 points, severe swelling and erythema. The points for each hind paw were added, and the sum was designated as the arthritis index. A single mouse could have a maximum arthritis index of 6.

To evaluate the extent of the functional disorder and/or ankylosis that occurred in the hind paws, each joint was scored from 0 (no functional change) to 3 points (severe functional disorder and/or ankylosis). Similar to the articular index, the points for each hind paw were added, and the sum was designated as the joint dysfunction index. The body weights of the infected and control mice were also monitored at regular intervals.

Bacterial growth in organs of infected mice. Organ infections in mice administered GAS JRS4 were determined by CFU evaluation 21 days after i.v. inoculation. The heart, lungs, liver, and knee joints of the hind paws were removed from mice with arthritis (arthritic index of $>$2) and placed in a tissue homogenizer with 1 ml of sterile phosphate-buffered saline (PBS). After the organ samples were homogenized, various dilutions were made in PBS and plated on THY agar plates containing 1 mg of streptomycin per ml. CFU were enumerated, and the results were expressed as number of CFU per whole organ.

Cytokine assays. Blood samples from mice infected with GAS JRS4 and from uninfected control mice were obtained before euthanasia on days 1, 3, 5, 10, and 20 after inoculation. Samples were incubated at 37°C for 1 h and then centrifuged, and the supernatants were stored at −80°C until use. Knee joints were obtained from mice infected or uninfected with GAS. Adjacent soft tissues were removed as much as possible and then homogenized in 1 ml of PBS containing 20 μl of protease inhibitor cocktail per ml (Sigma). The homogenized tissues were then centrifuged at 3,000 × g for 10 min, and the supernatants were stored at −80°C until analysis (35). TNF-α, IL-1β, and IL-6 levels in sera and joints were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Minneapolis, Minn.) according to the manufacturer’s recommendations. The RANKL concentrations in sera and joints were also measured with an ELISA kit (R&D Systems). The results are expressed as picograms per milliliter of serum or of joint homogenates. The detection limits of the assays were 3 pg/ml (each) for IL-1β and IL-6 and 5 pg/ml for TNF-α.

Serum sialic acid concentration. Serum samples were obtained from infected and uninfected mice 21 days after infection. The sialic acid concentration in

TABLE 1. Effect of S. pyogenes JRS4 inoculum size on arthritis in and mortality of CD-1 mice

<table>
<thead>
<tr>
<th>Inoculum size (CFU/mouse)</th>
<th>No. of mice</th>
<th>No. of mice that died&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of arthritic mice&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>4 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>2 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>25</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

<sup>a</sup> Up to 21 days after inoculation.

<sup>b</sup> Arthritis index ≥2 during 21 days.

FIG. 1. Characteristics of appearance of hind paws of female CD-1 mice infected with GAS strain JRS4. (A) Control hind paw (arthritis index = 0). (B) Swollen joint (arthritic index = 3) of a mouse on day 7 after i.v. infection of 2 × 10<sup>8</sup> CFU of GAS strain JRS4. (C) Appearance of arthritic joint on day 21. Severe abscesses are shown around the infected joint (arrowheads).
were designated as the bone destruction index. Scoring for bone destruction was acquired according to the following criteria: 0 points, no destruction; 1 point, mild loss of cortical or trabecular bone at a few sites; 2 points, moderate loss of bone at many sites; and 3 points, marked loss of bone at many sites. To evaluate the extent of bone destruction, sections were stained with hematoxylin and eosin. Scores for bone destruction were acquired according to the mean standard error, with \( P < 0.05 \) considered significant.

**RESULTS**

**Clinical course of arthritis.** The capacity of GAS strain JRS4 to produce a lethal infection was examined by i.v. inoculation of CD-1 mice with different numbers of bacteria. Table 1 shows that an inoculation of less than \( 2 \times 10^8 \) CFU of GAS JRS4 did not cause death, while the mortality rate increased to 80\% with an inoculum size of \( 4 \times 10^6 \) CFU per mouse. It was also noted that mice given \( 2 \times 10^8 \) CFU showed severe joint inflammation (Fig. 1B). The clinical signs of joint swelling were observed as early as 1 day after injection of \( 2 \times 10^8 \) CFU of GAS JRS4. The incidence of arthritis was greater than 80\% on day 5, and the most commonly involved joints were the ankles and wrists. Joint dysfunction was also evident in mice infected with \( 2 \times 10^8 \) CFU. At later time points, ankylosis and deposition of fibrous exudate in the articular cavities were observed. Furthermore, marked pus formation around the infected joints was seen (Fig. 1C). Inoculation with \( 5 \times 10^7 \) CFU of strain JRS4 did not cause death of mice, and DMEM-injected control mice did not exhibit arthritis or any extra-articular manifestations. In this regard, we found that GAS strain SSI-1 induced septic arthritis in CD-1 mice when more than \( 2 \times 10^8 \) CFU was inoculated, and infection with more than \( 1 \times 10^7 \) CFU resulted in death of mice within 7 days. However, infection with SSI-9 required more than \( 2 \times 10^7 \) CFU organisms to develop septic arthritis in CD-1 mice (data not shown).

The induction of joint arthritis was dependent on the number of GAS JRS4 CFU inoculated (Table 1). Mice inoculated with \( 5 \times 10^7 \) CFU showed mild articular swelling and erythema, and their arthritis index never exceeded a mean value of 1.5 throughout the observation period (Fig. 2B). In contrast, the arthritis index of mice inoculated with \( 2 \times 10^8 \) CFU reached 3 on day 11 (Fig. 2B). Inoculation with \( 2 \times 10^8 \) CFU resulted in a decrease in body weight (Fig. 2A), and severe ankylosis or joint dysfunction was also observed in those mice (Fig. 2C). Increasing the infection dose resulted in increased arthritis incidence, severity, and joint dysfunction.

**Histopathology and immunohistochemical staining.** The appearance of joints with an arthritis index of 3 on day 21 is shown in Fig. 1C. Histopathological analyses of knee joints of mice with an arthritis index of >2 showed synovial proliferation, marked infiltration of mononuclear cells, and polymorphonuclear leukocytes in the soft tissues (Fig. 3B and E). Destruction of both cartilage and subchondral bone was also found in scattered areas of the joints, and the articular cavities were filled with purulent exudate (Fig. 3B). Immunohisto-
chemical staining using anti-GAS antibodies revealed colonization of GAS strain JRS4 in the joints of infected mice. Bacteria were detected in both the articular cavities and bone marrow cavities. As shown in Fig. 3D, most of the organisms were colonized along the bone trabecula. In contrast, the joints of uninfected control mice were not stained by the anti-GAS antibodies (Fig. 3C).

Colonization of GAS strain JRS4 in various organs was also investigated on day 21 (Table 2). While numerous bacteria (10^7 CFU/joints) were found in the joints of infected mice with arthritis (arthritis index of >2), the number of GAS organisms in the heart, lungs, and liver was less than 10^4 CFU per organ.

**Kinetics of cytokine appearance.** Figure 4 shows that IL-1β concentrations in joints began to increase 3 days after injection of GAS strain JRS4 and reached a level of approximately 350 pg/ml. The concentrations of both of IL-1β and IL-6 in joints were higher than those of controls throughout the experimental periods. However, the cytokine concentrations in sera reached the maximal value on day 5, and a progressive decrease was observed from day 10 after injection (Fig. 4). TNF-α levels never exceeded 50 pg/ml in the joints (Fig. 4C) and remained lower than 10 pg/ml in serum. RANKL concentrations in both the joints and serum of infected mice were also assayed with an ELISA kit. The concentrations of RANKL in the joints were comparable to those of IL-6 and reached 200 pg (Fig. 5), while the concentrations in sera were below the detection limit. A progressive decrease in RANKL concentration in the joints was observed on day 21 after infection. We also measured serum sialic acid concentrations on day 21 and found a significant increase in infected mice (data not shown).

**Effect of OPG on bone destruction in septic arthritis.** Since a significant increase in RANKL in the infected joints was observed, we investigated the effect of OPG, a decoy receptor of RANKL, on GAS-induced bone destruction (Fig. 6). At the onset of arthritis, GAS-infected mice were administered OPG (1 mg/kg of body weight/day) subcutaneously daily from day 3 to day 9. On day 21, the mice were sacrificed, and the hind paws were examined for bone destruction. Inhibition of RANKL by OPG had no effect on the severity of inflammation (data not shown). However, OPG-
treated mice exhibited a reduction in loss of cortical and trabecular bone (Fig. 6C), whereas untreated infected mice developed severe bone lesions, characterized by partial to complete destruction of cortical and trabecular bone and erosion of the articular cartilages (Fig. 6B). Table 3 summarizes the effect of OPG treatment on GAS-induced arthritis. OPG-treated mice showed significant reduction of bone destruction.

DISCUSSION

We found in this study that infection with live GAS induced septic arthritis in mice. Most mice inoculated with $2 \times 10^8$ CFU of GAS strain JRS4 in DMEM (●) or with DMEM alone (□). Samples were obtained 1, 3, 5, 10, and 20 days after infection. Results are expressed as the mean ± standard error of three experiments. *, $P < 0.05$ versus uninfected control mice.

FIG. 4. IL-1β, IL-6, and TNF-α concentrations in sera and joints of CD-1 mice. Mice were injected with $2 \times 10^8$ CFU of GAS strain JRS4 in DMEM (●) or with DMEM alone (□). Samples were obtained 1, 3, 5, 10, and 20 days after infection. Results are expressed as the mean ± standard error of three experiments. *, $P < 0.05$ versus uninfected control mice.

<table>
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<tr>
<th>Organ</th>
<th>S. pyogenes JRS4 recovered (CFU/tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joint</td>
<td>($1.03 \pm 0.87) \times 10^7$</td>
</tr>
<tr>
<td>Lung</td>
<td>($4.36 \pm 3.89) \times 10^3$</td>
</tr>
<tr>
<td>Heart</td>
<td>($2.33 \pm 4.04) \times 10^2$</td>
</tr>
<tr>
<td>Liver</td>
<td>($5.66 \pm 4.04) \times 10^2$</td>
</tr>
</tbody>
</table>

* Samples were obtained from mice with severe arthritis (arthritis index = 3) 21 days after infection. Results are expressed as the mean ± standard deviation ($n = 3$). $P < 0.05$ versus CFU in joint (Mann-Whitney U test).
CFU of GAS strain JRS4 suffered septic arthritis. This may be a good experimental model of septic arthritis induced by GAS. To study the pathogenesis of septic arthritis, a similar murine model was developed using S. aureus (5, 33) and GBS (24, 34). Certain virulence factors such as superantigens and exotoxins have been implicated in S. aureus-induced arthritis (33). Since GAS produces several kinds of virulence factors, such as superantigens and exotoxins, these factors may contribute to the pathogenesis of GAS arthritis. It was known that cell wall components of GAS induced experimental arthritis in strains of rats (6). However, induction of this type of streptococcal cell wall arthritis is difficult in experimental mice, and i.v. injection of GAS cell wall components induced acute arthritis in BALB/c and DBA/1J mice (6, 15). Acute swelling and erythema of the ankles and wrists reached maximum severity within a few days after injection; however, histological studies showed that there was no pannus formation or destruction of cartilage and subchondral bone in the mice (15). These pathological features are quite different from those of the septic arthritis shown in this study.

It was reported that synovial fluid cultures were bacterially positive in 90% of nongonococcal bacterial arthritis cases (10). Recent studies have shown that GAS can attach to and invade several types of eukaryotic cells, such as epithelial cells and keratinocytes (1, 7, 13, 14, 19, 21). Furthermore, entry of GAS into various host cells has been shown to occur in vivo and may contribute to bacterial persistence despite antibiotic therapy (22). Several investigators have suggested the need for chronic penicillin prophylaxis in patients with poststreptococcal reactive arthritis (20), and it was also reported that >50% of patients suffering from such arthritis were positive for GAS in throat cultures (3). Therefore, GAS can invade joint tissues, and its persistence may induce not only septic arthritis but also poststreptococcal reactive arthritis. In fact, there was chronic colonization of GAS in the joints of infected mice in the present study. GAS colonization in the joints may directly trigger tissue damage by producing several tissue-damaging enzymes, such as proteases (7), as well as superantigens, such as SpeA, which eventually stimulate host immune systems. The present histological examinations showed that infiltration of mononuclear cells in the infected joints occurred in GAS-induced arthritis (Fig. 3). These cells produce inflammatory cytokines, such as IL-1 and TNF-α, which in turn also induce the destruction of bone and cartilage in joint tissues.

Cytokines play pivotal roles in inflammation and immune responses. We found high levels of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, in the joints of mice infected with GAS. Tissi et al. (35) also showed an increase in the levels of IL-1, IL-6, and TNF-α in serum as well as locally in GBS-induced septic arthritis, and they revealed that systemic inoculation with pentoxifylline, an inhibitor of IL-1β and IL-6 production, resulted in a decrease or abolition of cytokine production, along with a consequent reduction in both the incidence and severity of GBS arthritis. Regarding mouse septic arthritis caused by S. aureus, TNF and lymphotoxin double-mutant mice are resistant (12). These results suggest the participation of proinflammatory cytokines in septic arthritis caused by GAS, GBS, and S. aureus.

RANKL is a newly discovered key mediator of osteoclast differentiation and bone resorption (11, 30). Several investigations have revealed the involvement of RANKL in the bone destruction in rheumatoid arthritis. Kotake et al. (18) first reported that increased concentrations of RANKL were detected in synovial fluid from patients with rheumatoid arthritis.

![RANKL concentration graph](image)

**FIG. 5.** RANKL concentrations in sera and joints of GAS-infected CD-1 mice. Mice were injected with 2 x 10^8 CFU of GAS strain JRS4 in DMEM ( ■ ) or with DMEM alone ( □ ). Samples were obtained at 1, 3, 5, 10, and 20 days after injection. Results are expressed as the mean ± standard error of three experiments. *, P < 0.05 versus uninfected control mice.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of mice</th>
<th>No. of mice with bone destruction^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>No infection</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><em>S. pyogenes</em> JRS4 infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Treated with OPG^c</td>
<td>5</td>
<td>1</td>
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</table>

^b Bone destruction index greater than 2. P < 0.05 versus untreated. The results for no infection versus treated with OPG were not significant (Mann-Whitney U test).

^c OPG (1 mg/kg/day) was administered subcutaneously daily from day 3 to day 9 after GAS infection.
in vitro infection of GAS induces RANKL expression in mouse osteoblasts (23), suggesting that the colonization of GAS in joints induces local RANKL production from osteoblasts.

Several investigations have shown a possible link between the pathogenesis of rheumatoid arthritis and bacterial components. Both bacterial DNA and peptidoglycan have been detected in the joints of some patients with rheumatoid arthritis (9, 36). Here we observed an increase in serum sialic acid, a marker of rheumatoid arthritis (29), in GAS-infected mice. Experimental rats with adjuvant arthritis showed a similar increase in serum sialic acid (32). Therefore, some common mechanisms may underlie both septic arthritis and rheumatoid arthritis.

In summary, we have demonstrated that i.v. inoculation of mice with GAS induces experimental septic arthritis and local production of RANKL is involved in bone destruction in the infected joints.

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