Role of Interleukin-6 in the Pathogenesis of an Avian Model of *Staphylococcus aureus* Arthritis


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ABSTRACT

To evaluate the role of interleukin-6 (IL-6) in arthritis induced by *Staphylococcus aureus*, a chicken model was developed for study. A total of 120 healthy broilers (8 wk old) were randomly divided into 4 groups. Two groups were injected with 0.35 mL of *Staph. aureus* (7.1 × 10⁹ cfu/mL) into the right hock joints and the other 2 were injected with 0.35 mL of sterile saline into the same joints. One group of each of the 2 treatment groups was fed levofloxacin at a dose of 5 mg/kg of BW on the third day postinoculation for 4 successive days. Chicken blood samples were obtained on d 0, 1, 4, 7, 14, 21, 28, and 35 postinoculation. Chicken IL-6 (chIL-6) activities and concentrations in serum were quantified by B9 bioassay and human IL-6 ELISA, respectively. The results showed that chIL-6 activities and concentrations were reduced (P < 0.05) in the serum of infected broilers treated with levofloxacin compared with birds injected only with *Staph. aureus*. Levofloxacin treatment had no effect on IL-6 activities and concentrations in uninfected broilers. There was a strong correlation (r = 0.91) between serum chIL-6 activities by the B9 bioassay and serum IL-6 concentrations determined by the human IL-6 ELISA. We concluded that chIL-6 is involved in the progression of chicken arthritis induced by *Staph. aureus*, and that it contributes to disease incidence and mortality.

Key words: interleukin-6, levofloxacin, broiler, *Staphylococcus aureus*, arthritis

INTRODUCTION

*Staphylococcus aureus* is an important human and veterinary pathogen that causes great economic loss in the poultry industry. The diseases associated with this bacterium vary in severity from superficial skin and ophthalmic infections to life-threatening endocarditis, septic arthritis, and septicemia. Typically, staphylococcal arthritis is a hematogenously spread disease that preferentially affects already-infected joints and causes irreversible joint destruction and significant mortality; it is most prevalent in 6- to 12-wk-old broiler breeders. Despite antibiotic treatment, which is effective in eradicating the bacteria, the reported mortality from staphylococcal arthritis in chickens ranges from 3 to 20% (Huang et al., 2002).

The incidence of *Staph. aureus* arthritis in humans ranges from 0.002 to 0.01% (Goldenberg, 1999). In most cases, human staphylococcal arthritis has been studied using mice or rabbits as experimental animal models (Bremell et al., 1992; Smith et al., 1997). Moreover, experimental chicken models have been developed (Zhu et al., 1990; Wei et al., 1995; Cheng et al., 2003), which have shown that *Staph. aureus* arthritis in chickens closely resembles the human disease in pathogenesis (Alderson et al., 1986). Studies of chicken models of staphylococcal arthritis may overcome the limitations encountered in humans, such as disease model establishment and tissue sample collection from the synovial membrane and cartilage.

*Staphylococcus aureus*-induced arthritis is associated with an inflammatory response, and a number of studies have investigated the role of inflammatory cytokines in the infectious process (Bremell et al., 1992; Nakane et al., 1995). Interleukin-6 is one of the most pleiotropic interleukins released at sites of injury or infection (Verdrengh and Tarkowski, 1997). Some investigators have reported that IL-6 can activate osteoclasts and that its release increases the damage of joints during the arthritic process (Green et al., 1994). Increased serum IL-6 levels have been observed throughout the course of *Staph. aureus* arthritis in several animal models (Bremell et al., 1992; Green et al., 1994). The production of IL-6 has been shown to be highly correlated with death, both in patients with *Staph. aureus* arthritis and in animal models of the disease (Brouckaert and Fiers, 1996), but there have been only a few reports on the production of IL-6 during *Staph. aureus* arthritis in chickens. Thus, the present study was undertaken to investigate the role of IL-6 in the pathogenesis of *Staph. aureus* arthritis in a chicken model.
MATERIALS AND METHODS

Animals

Five-week-old avian broilers were obtained from Chia Tai China Ltd. (Wuhan, China) and were maintained in the animal facility of the Department of Veterinary Medicine, Huazhong Agricultural University. They were housed in isolation in wire-floored cages under standard conditions of light and temperature, and were fed a standard laboratory chow and water ad libitum.

Bacterial Strains

The Staph. aureus strain used in this study was originally isolated from a swollen hock joint of a spontaneously arthritic broiler (Huang et al., 2002). This bacterial strain was shown to be β-hemolysin-, catalase-, and coagulase-positive. The bacteria were cultured on blood agar (5% sheep erythrocytes) for 24 h at 37°C and then re-incubated on nutrient agar medium for 18 h at 37°C before inoculation. From the culture, a bacterial suspension was prepared in saline at a concentration of $7.1 \times 10^9$ cfu/mL. Viable counts were used to check the concentration of bacteria injected.

Cell Culture

Murine hybridoma cell line B13.29, which is dependent on IL-6 for growth, has previously been described (Tomas et al., 1992). For IL-6 determination, the more sensitive subclone B9 was used (Diamant et al., 1994). The B9 cells (Laboratory of Animal Virology, Huazhong Agricultural University) were seeded at 2.0 × $10^3$ cells/mL and grown at 37°C, 5% CO$_2$ in Roswell Park Memorial Institute-1640 medium (RPMI-1640, catalog no. AML17586, HyClone, Logan, UT) containing 1% L-glutamine, 10% fetal bovine serum (catalog no. SH30087, HyClone, Logan, UT) 0.05 mM 2-mercaptoethanol, 100 units/mL of penicillin (catalog no. 2003064, Amresco, Solon, OH), and 1 mg/mL of streptomycin (catalog no. 20030315, Amresco). During routine culture, recombinant IL-6 (rhIL-6, lot no. 02016, PeproTech, Rocky Hill, NJ) was added at 5 units/mL.

Experimental Protocol

At 8 wk of age, the chickens were randomly divided into 4 groups of 30 broilers. Two groups were injected with 0.35 mL of Staph. aureus suspension ($7.1 \times 10^9$ cfu/mL) in sterile saline intra-articularly into the right hock joints on d 0, and the other 2 groups were injected in the same way with 0.35 mL of sterile saline. One group in each of the 2 treatment groups was fed levofloxacin (LVFX, catalog no. 20030406, Daming Co., Ltd., Henan Province, Zhengzhou, China) at a dose of 5 mg/kg of BW on the third day post Staph. aureus/saline inoculation (PID) for 4 successive days. Blood samples, 3 mL per broiler, were obtained via the wing-web at selected intervals (0, 1, 4, 7, 14, 21, 28, and 35 PID, 8 broilers per time point), centrifuged, then aliquoted equally and stored at −40°C for IL-6 bioassay and ELISA.

Histopathological Examination

The birds were killed 35 d after inoculation of bacteria. After routine fixation, decalcification, and paraffin embedding, the joints were examined histologically for synovial hypertrophy (synovial membrane thickness of more than 2 cell layers), pannus formation (joint cartilage covered with synovial tissue), and destruction of cartilage and subchondral bone.

Chicken IL-6 Bioassay

The chicken IL-6 (chIL-6) activities in serum samples were measured according to their ability to promote growth of the IL-6-dependent murine hybridoma B9 cells (Helle et al., 1991; Rath et al., 1995). The rhIL-6 standard and serum samples (incubated at 56°C for 30 min) were serially diluted (2-fold steps) with RPMI-1640 medium. One hundred microliters of serially diluted serum samples and rhIL-6 standard were added in triplicate to cell cultures at a concentration of $10^4$ cells/well for IL-6 bioassay and ELISA.

chIL-6 ELISA

A human IL-6 ELISA kit (Laboratory of Immunology, The Fourth Military Medical University, Xian, China) was used to determine chIL-6 concentrations in serum samples according to the manufacturer’s instructions. Briefly, monoclonal antibodies had been applied to 96-well microtiter plates (Nunc, Rochester, NY) by the manufacturer. The rhIL-6 standard and serum samples were added, followed by 2-fold serial dilutions (20 μL/well), and anti-human monoclonal antibody conjugated with biotin (1:100) was added and incubated for 2 h at 25°C. Plates were then washed with washing buffer (PBS-0.05% Tween 20) 4 times for 3 min, and avidin conjugated with horseradish peroxidase was added and incubated for 30 min at 25°C. After washing as described above, the color
was developed with 3,30,5,50-tetramethyl benzidine (TMB, Sigma) for 10 min and the reaction was stopped with 2.5 N H2SO4. Extinction at 450 nm was measured with a universal microplate reader (ELX800, BioTek, Winooski, VT).

Statistical Analysis

The SPSS program version 12.0 was used for data analysis (Zhang et al., 2005; Lu et al., 2006). Linear correlations between serum chIL-6 activities, as determined by the B9 bioassay, and serum IL-6, as determined by the human IL-6 ELISA, were analyzed. Activities or concentration differences were compared between 2 random groups at the same time point and were compared with d 0 in the same group by 2-way ANOVA. In all cases, differences were considered significant if P ≤ 0.05.

RESULTS

Clinical Course of Infectious Arthritis

Within 2 d after injection of Staph. aureus, the infected birds displayed depression, huddling, and fever. Feed and water consumption as well as weight gain were significantly reduced. Birds were lame and showed a reluctance to move. At PID 7, the arthrocele of the injected (right) hock became apparent and the left hock developed an arthrocele also. The clinically observed maximum frequency of arthritis was 100% (30/30) in Staph. aureus-injected birds as compared with 83.3% (26/30) in Staph. aureus-injected birds treated with LVFX at PID 14. Throughout the experiment, 23.3% (8/30) birds in the Staph. aureus-injected, no-LVFX-treatment group died. The cross-clinical signs of infected birds treated with LVFX improved gradually, and only 3.3% (1/30) died in the experiment. Histopathological examination confirmed the clinical observations. Lesions of infected birds consisted of swollen joints containing serous or caseous exudates at PID 35. The frequency of synovitis in Staph. aureus-injected, no-LVFX birds was 83.3% (25/30) as compared with 60% (18/30) in Staph. aureus + LVFX-injected birds. Ninety percent (27/30) of Staph. aureus-injected, no-LVFX birds displayed cartilage destruction, compared with 70% (21/30) in Staph. aureus-injected birds treated with LVFX. The birds injected with saline as well as those injected with saline + LVFX did not exhibit arthritis or any extra-articular manifestations during the experiment.

Serum chIL-6 Activity

With the B9 bioassay, serum chIL-6 activities in the Staph. aureus-injected group began to grow from a mean of 4.08 to 9.24 unit/mL at PID 1 (P < 0.01) and reached a maximal mean value of 23.25 unit/mL on PID 14 (P < 0.01). Serum chIL-6 activities in the Staph. aureus-injected group were significantly higher than those in the saline-treated group (P < 0.05 at PID 1, 4, and 28; P < 0.05, and P < 0.01, at PID 7, 14, and 21). In the Staph. aureus + LVFX-treated group, serum chIL-6 activities were slightly reduced (3.90 unit/mL), but increased at PID 14 and reached the maximal mean value of 7.23 unit/mL on PID 28 (P < 0.01) compared with at d 0, and compared with Staph. aureus-injected birds (P < 0.05 at PID 4 and 7; P < 0.01 at PID 14 and 21), but there was no significant difference in serum chIL-6 levels between the broilers injected with saline and those uninfected and treated with LVFX (Table 1).

Seum chIL-6 Concentrations

Table 2 shows that serum chIL-6 concentrations in the Staph. aureus-injected birds rose slowly from 0.15 ng/mL at d 0 and peaked at a mean value of 3.60 ng/mL at PID 14 (P < 0.01), whereas chIL-6 concentrations in saline-treated birds retained a mean value of approximately 0.16 ng/mL. There was a significant difference in serum chIL-6 concentrations in infected birds, as compared with concentrations in the saline-treated group (P < 0.05 at PID 1 and 35; P < 0.01 at PID 4, 7, 14, 21, 28). In the Staph. aureus + LVFX-treated group, chIL-6 concentrations were inconsistent, increasing from 0.15 ng/mL at d 0 to 0.24 ng/mL at d 4 and decreasing to 0.18 ng/mL at d 14 (P < 0.05 at PID 7, 28, and 35; P < 0.01 at PID 4, 14, and 21) compared with birds injected only with Staph. aureus. There was no significant difference in serum chIL-6 levels between the 2 groups of saline injection only and saline + LVFX.

Correlation Analysis

During the experiment, the changes in serum chIL-6 activities detected by bioassay showed a strong correlation (r = 0.91) with serum chIL-6 concentrations determined using the human IL-6 ELISA.

DISCUSSION

Role of chIL-6 in Staph. aureus Arthritis

Interleukin-6 is a multifunctional cytokine that plays a major role in regulating immune responses and acute-phase reactions (Kishimoto et al., 1995). Interleukin-6 is produced by many different cell types and acts on B lymphocytes (Hirano et al., 1986), T lymphocytes (Housiaux et al., 1988), hepatocytes (Gauldie et al., 1987), hematopoietic progenitor cells (Ikebuchi et al., 1987), and cells of the central nervous system (Satoh et al., 1988). Circumstantial evidence has suggested that a functional homologue of mammalian IL-6 exists in chickens (Samad et al., 1993; Rath et al., 1995; Schneider et al., 2001).

In the present study, enhanced systemic production of chIL-6 in serum was observed in infected birds when compared with saline-treated birds, similar to the report of Bremell et al. (1992) in a murine model of staphylococcal arthritis. However, the Staph. aureus-infected birds treated with LVFX during the experiment showed reduced levels of chIL-6 in serum, as compared with the
### Table 1. Kinetics of serum chicken interleukin-6 (chIL-6) activities in broilers at different time points after inoculation of *Staphylococcus aureus* (n = 8 per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Activities of chIL-6 at days after inoculation (units/mL)</th>
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<tbody>
<tr>
<td></td>
<td>0 d</td>
</tr>
<tr>
<td>Saline</td>
<td>4.08 ± 0.78</td>
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<tr>
<td>Saline + levofloxacin</td>
<td>4.08 ± 0.78</td>
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<tr>
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*Comparison with the saline-treated group (P < 0.05).*
*Comparison with the saline + levofloxacin-treated group (P < 0.05).*
*Comparison with the *Staph. aureus*-treated group (P < 0.05).*
*Comparison with the *Staph. aureus*-treated group (P < 0.01).*
*IL-6 activities different time after inoculation compared with that before inoculation (d 0; *P < 0.05; **P < 0.01).*
*ND = not done.*

### Table 2. Kinetics of serum chicken interleukin-6 (chIL-6) concentrations in broilers at different time points after inoculation of *Staphylococcus aureus* (n = 8 per group)

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<tr>
<td></td>
<td>0 d</td>
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<tr>
<td>Saline</td>
<td>0.15 ± 0.04</td>
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<td>Saline + levofloxacin</td>
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*Comparison with the saline + levofloxacin-treated group (P < 0.05).*
*Comparison with the *Staph. aureus*-treated group (P < 0.05).*
*Comparison with the *Staph. aureus*-treated group (P < 0.01).*
*Interleukin-6 concentrations at different times after inoculation compared with that before inoculation (d 0; *P < 0.05; **P < 0.01).*
*ND = not done.*
Staph. aureus-injected birds. At the same time, chIL-6 levels in the saline-treated group were similar to those in the saline + LVFX-treated group, which indicates that LVFX did not affect endogenous levels of chIL-6. Taken together, these data show a strong involvement of chIL-6 in the pathogenesis of Staph. aureus arthritis in broilers and that it plays a detrimental, damaging role in the infectious process of Staph. aureus arthritis.

Interleukin-6 exerts its effect by binding specific receptors on the cell surface. Interleukin-6 produced by osteoblasts has been shown to modulate the activity of bone-resorptive osteoclasts directly or indirectly, resulting in induction of osteoclast differentiation or osteoclast-mediated bone demineralization (Ishimi et al., 1990; de la Mata et al., 1995; Greenfield et al., 1995). It has also been demonstrated that the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 in serum are associated with the severity of the infectious process. Additionally, IL-1β and TNF-α can induce chondrocytes and synovial macrophages to release a high production of IL-6 locally in bone, which consequently increases damage to joints during the arthritic process (Green et al., 1994). In our trial, the chIL-6 level of Staph. aureus-infected chickens rose quickly and the mortality of chickens reached 23.3%; yet the chIL-6 level of Staph. aureus-infected + LVFX-treated chickens remained consistent and the mortality of chickens was decreased (3.3%); hence, we deduced that chIL-6 may be involved in the pathogenesis of S. aureus arthritis as injury in chickens.

Assay Methods of chIL-6

Many different assay systems are available for quantifying the levels of mammalian IL-6 or for detecting cells that express the IL-6 gene. To date, no reports have been found on a standard ELISA for chIL-6, yet recombinant chicken IL-6 was commercially available (www.serotec.com, 2001). Recombinant murine IL-6 or rhIL-6 standards have also been adopted in the quantification of chIL-6 levels with bioassays to establish dose-response curves (Rath et al., 1995; Lynagh et al., 2000; Xie et al., 2000). A functional homologue has been shown to exist between chIL-6 and mammalian IL-6 (Samad et al., 1993; Rath et al., 1995; Schneider et al., 2001). In a previous study (Cheng et al., 2003), we used a human IL-6 ELISA kit to quantify chIL-6 levels during Staph. aureus arthritis.

A standard bioassay for IL-6, which exploits the fact that IL-6 has a proliferative effect on IL-6-dependent B9 cells, has been used to determine whether IL-6 is active (Rath et al., 1995; Lynagh et al., 2000; Xie et al., 2000; Schneider et al., 2001). Rath et al. (1995) first used IL-6-dependent B9 cells and an MTT colorimetric assay to detect chIL-6-like factors in the sera and ascite fluids of chickens. In this study, we measured chIL-6 activities in the sera of healthy broilers, and the average chIL-6 activity values were 4.33 ± 0.75 unit/mL. Similar results were obtained in other studies (Lynagh et al., 2000). Our results showed a positive correlation (r = 0.91) between the ELISA and the B9 bioassay when chIL-6 in serum was measured. These results indicate that a human IL-6 ELISA kit can be used to quantify chIL-6 in the sera of chickens.

Effect of LVFX Treatment on Staph. aureus Arthritis

Levofloxacin, a member of the fluoroquinolone family, possesses excellent potent activity against a wide microbial spectrum. Hu et al. (2002) showed that LVFX was effective in controlling avian staphylococcosis attributable to Staph. aureus when administered in drinking water.

Treatment of LVFX started at PID 3 because broilers showed clinical symptoms at the time, and the doses of LVFX were chosen according to earlier studies (Hu et al., 2002). In the present study, the infected birds treated with LVFX during the experiment showed reduced levels of chIL-6 in serum, as compared with Staph. aureus-injected birds. At the same time, most of the Staph. aureus-injected birds exhibited less severe clinical signs and lesions after receiving LVFX treatment. Brouckaert and Fiers (1996) demonstrated in other experimental animal models that the production of IL-6 is highly correlated with disease severity and mortality. Our previous study suggested that the Staph. aureus strain used in this experiment was very susceptible to LVFX (Li et al., 2004). The reduction of live Staph. aureus in chickens induced a decrease in exotoxin production, which in turn stimulated a reduced production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (Bremell et al., 1992; Nakane et al., 1995; Thijss et al., 1996). Therefore, blockage of IL-6 hypersecretion in vivo may be developed as a therapeutic application against Staph. aureus arthritis. In conclusion, the present study clearly demonstrated that chIL-6 participates in the infectious process of Staph. aureus arthritis in broilers and contributes to the pathogenesis of the disease.

ACKNOWLEDGMENTS

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REFERENCES