Correlation of Histopathologic and Bacteriologic Changes with Cytokine Expression in an Experimental Murine Model of Bacteremic Staphylococcus aureus Infection

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Staphylococcus aureus infections are often life threatening. Relatively little is known about the host response to these infections, in particular, the role played by cytokines. We established a mouse model of bacteremic S. aureus infection to correlate bacteriologic findings and pathologic changes with cytokine gene expression. Bacterial density in blood and tissue was highest at 1 h and minimal by 48 h. Despite the rapid clearance of bacteria, pathologic abnormalities and inflammatory cytokines were detected after clearance of the bacteria. The number of infiltrating inflammatory cells, as well as the size of inflammatory foci, increased with time. Intestinal accumulation of inflammatory cells and tissue damage, such as microabscesses, edema, and necrosis progressed following clearance of bacteria from the tissues. Levels of tumor necrosis factor and interleukin-1 protein in serum were detectable at 1 h and peaked at 4 h. Interleukin-6 protein expression showed different kinetics, with low levels detected at 1 h and increasing levels at 72 h postinfection. Tumor necrosis factor and the interleukins were expressed in inflammatory and noninflammatory cells in lung, liver, and heart tissues. Leukocytes in the infected tissues were highly reactive with antibodies to the three cytokines, suggesting that activated leukocytes are a major source of inflammatory cytokines after staphylococcal infection. Expression of interleukin-1 and interleukin-6 in tissue-specific cells and endothelial cells was also detected in infected tissues, indicating that cells other than leukocytes contribute to the elevated cytokine levels in this model. Once initiated, expression of inflammatory cytokines contributes to the pathogenesis of S. aureus disease.

Despite the availability of effective antimicrobial agents, Staphylococcus aureus continues to cause life-threatening infections, including septic shock (2, 24, 33). While a great deal is known about the inflammatory profile that occurs in response to endotoxin during gram-negative bacterial infections, surprisingly little is known about the host response to staphylococcal septicemia or the bacterial components that initiate this response (2, 32). It has been reported that levels of the cytokines tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6 and IL-8 in serum are correlated with the severity of systemic bacterial infections in humans (4, 5, 15). A similar pattern of cytokines can be elicited by endotoxin alone in experimental animal models of infection (9, 18). A limited number of studies have examined the cytokine response to S. aureus infections (16, 21, 31).

In vitro studies have demonstrated that cytokine gene expression is induced in monocytes following infection with S. aureus cellular components, including peptidoglycan, lipoteichoic acid, or enterotoxin (1, 10, 23, 27, 28). Endothelial cells express IL-1, IL-6, and IL-8 following S. aureus infection (35, 36).

In the present study, we examined the cytokine most closely associated with mortality—TNF, IL-1, and IL-6. The purpose of this investigation was to demonstrate that an experimental mouse model of bacteremic S. aureus infection could be used to correlate cytokine activity in serum and tissue with histopathologic findings and the density of bacteria in blood and tissue.

MATERIALS AND METHODS

Preparation of bacteria. Wb, a clinical isolate from a patient with endocarditis used in previous studies, was selected for this study (13, 29, 36). Bacteria were stored in nutrient broth (Difco Laboratories, Detroit, Mich.)–15% glycerol at −70°C and subcultured on blood agar. Fresh colonies were inoculated into Todd-Hewitt broth (BBL, Cockeysville, Md.) and grown overnight at 37°C. The bacteria were collected by centrifugation, washed, and resuspended in saline at a concentration of 5 × 10^8 CFU/ml measured spectrophotometrically at a wavelength of 620 nm prior to injection. The bacterial inoculum was confirmed by colony counting.

Animals and infection. Groups of at least four Swiss-Webster female mice (8 to 10 weeks old) were used. Mice were injected intravenously via the tail vein with Wb (10^7 CFU/mouse) in 0.2 ml of saline. Control mice were injected with the same volume of saline. Three independent sets of experiments were performed. All procedures were approved by the institutional animal care and use committee. Animal housing and care were in accord with the guidelines established by the National Institutes of Health.

Collection of blood and tissues for determination of bacterial density and cytokine levels. At different times (1, 3, 5, 6, 10, and 12 h) after infection, mice were anesthetized by inhalation of Methofane (methoxyflurane; Pitman-Moore, Mundelein, Ill.) and subcutaneously on blood agar. Fresh colonies were inoculated into Todd-Hewitt broth (BBL, Cockeysville, Md.) and grown overnight at 37°C. The bacteria were collected by centrifugation, washed, and resuspended in saline at a concentration of 5 × 10^8 CFU/ml measured spectrophotometrically at a wavelength of 620 nm prior to injection. The bacterial inoculum was confirmed by colony counting.

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Determination of cytokine levels in serum. IL-1 and TNF proteins from the sera of infected mice were assayed by enzyme-linked immunosorbent assay (ELISA) (ELISA kit; Endogene, Cambridge, Mass.). IL-6 protein from sera of Wb-infected mice was assayed using the IL-6-dependent cell line 7TD1 (obtained from J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium)
S.M.F. on a scale of 0 to 11.Interstitial inflammation or microabscesses; 11, more extensive abscess formation; 1, no inflammation; +, focal interstitial inflammation; ++, more diffuse interstitial inflammation; ++++, intense interstitial inflammation or microabscesses; +++++, more extensive abscess formation with tissue necrosis). The pathologist was unaware of the treatment group.

**RESULTS**

**Bacterial density in blood and tissues following intravenous injection of S. aureus.** Mice were given an intravenous injection of 10⁷ CFU of *S. aureus* (Wb). Clinically, the infected mice became inactive, huddled together in the cage, and were sweating; there were no deaths during the 72-h period of analysis. Blood samples were collected at different time periods (15 min to 72 h). Bacteria were rapidly cleared from the blood, with the greatest bacterial density observed 15 min after injection. By 48 h, few bacteria were cultured from the blood (Fig. 1A). No cultures from the blood or tissues were sterile at 1 or 4 h. The blood was sterile (≤10 colonies/100 μl) in one of five mice at 24 h and in five of five mice at 72 h. The liver and spleen were sterile in two of five mice (≤5 colonies/mg) at 24 h and in five of five mice at 48 h.

**Histopathologic changes following S. aureus intravenous infection.** Tissue sections of the lung, heart, and liver collected from mice at different time points after intravenous staphylococcal injection (1, 4, 24, 48, and 72 h; four or five animals per time point) were examined (Fig. 2). The pattern and degree of tissue damage varied from animal to animal. Some tissue sections displayed focal damage, while others showed a diffuse inflammatory response. Increased accumulation of inflammatory cells in the lung, heart, and liver was observed by 4 h after infection. These changes were noted in the liver at 1 h. While the heart showed less leukocyte accumulation at 4 h than the lung and liver, extensive inflammation was detected by 24 h (three animals) with abscess formation. Figure 3 shows H & E and Gram staining of the same section containing an abscess with a large collection of bacteria in the myocardium.

Tissue injury was more advanced at 48 and 72 h in the lung, heart, and liver (Fig. 2, 48 h). The histopathologic changes included leukocytes migrating away from vessels into the tissue parenchyma, accumulation of inflammatory cells in the interstitial spaces, and evidence of interstitial edema in the lung, heart, and liver. The presence of a large number of dispersed leukocytes, as well as aggregates of inflammatory cells, in these tissues increased with time (Fig. 2).

Small foci of inflammatory cells or microabscesses were seen in the livers of all at the time points examined. Vacuolization indicative of tissue injury was seen in the liver at 24 h. Abscesses were present in the infected hearts after 24 h (data not shown). The damage to the lungs was extensive, with focal pneumonitis and interstitial and intraalveolar edema beginning at 24 h and detected at 48 and 72 h (Fig. 2).

In summary, inflammatory cells were first seen within vessels and in the adjoining parenchymal tissue. Other changes, including interstitial edema, formation of microaggregates of leukocytes within the tissue parenchyma, and tissue necrosis,
were seen at 48 h or later. The degree of tissue inflammation and damage in the different tissues is shown in Table 1.

**Cytokine levels in serum after intravenous infection with *S. aureus***. Blood samples from *S. aureus*-infected mice were assayed for levels of TNF, IL-1, and IL-6 in serum. Levels of TNF and IL-1 in serum were highest 4 h after injection and decreased thereafter (Fig. 4). Low levels of TNF were still detected in the serum after 24 h (11 ng/ml). A rapid decrease of IL-1 in serum was observed at 24 h (7 ng/ml). IL-6 levels in serum differed from those of TNF and IL-1. IL-6 was detected...
as early as 1 h after injection and continued to increase until 72 h, the last time point assayed (Fig. 4).

Expression of cytokines in tissue after intravenous infection with S. aureus. Lung, heart, and liver tissue sections from infected mice (four animals at each time point) were examined by immunocytochemistry to assess cytokine gene expression in the tissues. The staining patterns and intensity of expression of these cytokine proteins varied in the different tissues and at the different time points. Expression of all three cytokines was observed in all of the infected tissues examined. IL-1, TNF, and IL-6 proteins were not detected in any of the uninfected tissues at the time points tested. Figure 5 (uninfected) shows an example of uninfected tissue treated with antibodies to TNF. Additionally, the infected tissues from different time points exhibited no reactivity with a negative control rabbit immunoglobulin G antibody (data not shown).

Patterns of TNF expression in tissues. TNF expression (by different cell types) was noted in all tissue sections of the infected lungs, hearts, and livers (Fig. 5). TNF protein was detected in all of the lungs at 1 h. Low levels of TNF were also present in the heart and liver at this time point. The most intense reactivity for TNF was observed at 24 h. Although expression was decreased by 48 and 72 h, positive TNF reactivity of inflammatory cells and tissue-specific cell types, such as Kupffer cells and alveolar lining cells, was still detected at these time points. TNF-expressing inflammatory cells were detected in blood vessels and tissue spaces at all time points (Fig. 5). Intense TNF reactivity of inflammatory cells within the alveoli and alveolar lining cells was detected after 4 h (Fig. 5, TNF, lung). Expression of TNF by intravascular inflammatory and interstitial cells in the heart was also observed (Fig. 5, heart).

Patterns of IL-1 expression in tissues. IL-1, like TNF, was detected in all of the organs examined. IL-1 protein was first detected in the lung 1 h after injection. Focal expression of IL-1 was observed in most tissues 4 h after injection, with the intensity increased at this time. Diffuse IL-1 protein expression was detected in most sections of the lung, heart, and liver at 24 and 48 h (Fig. 5). By 72 h after infection, IL-1 expression decreased in all tissues.

IL-1 expression by inflammatory, alveolar lining, and endothelial cells was first seen in the lung. Expression of IL-1 by endothelial cells in blood vessels was noted after infection. Although arterial endothelial cells showed no reactivity with IL-1 antibody in 4-h lung and heart samples, IL-1 in endothelial cells of thin-walled vessels compatible with veins was detected (Fig. 5, IL-1, lung). Expression of IL-1 by intravascular and interstitial inflammatory cells was present at all of the time points in all of the tissues (Fig. 5, IL-1, heart). Kupffer cells and sinusoidal lining cells around the central vein in the liver also expressed IL-1 (Fig. 5, IL-1, liver).

Patterns of IL-6 expression in tissues. The pattern of IL-6 expression was not comparable to that of either IL-1 or TNF (Fig. 5). IL-6 protein was detectable in the lung, heart, and liver as early as 1 h. Increased levels of IL-6 protein in all of the tissues were observed until 24 h and remained at similar intensities for up to 72 h.

Inflammatory cells within intravascular and infiltrating cells

<table>
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<tr>
<th>Time (h) postinfection</th>
<th>Organ</th>
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<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
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<td>0</td>
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<tr>
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<td>++</td>
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<td>++</td>
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<td>0</td>
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<tr>
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* The results are for four animals per time point. Scale: 0, no inflammation; +, focal interstitial inflammation; ++, more diffuse interstitial inflammation; ++++, intense interstitial inflammation or microabscesses; ++++, more extensive abscess formation with tissue necrosis (abscess); ND, not determined.

FIG. 4. Levels of TNF, IL-1, and IL-6 proteins in serum after S. aureus infection. Blood samples from infected mice were collected at different time points after injection of Wb bacteria. The sera were assayed for TNF or IL-1 by ELISA (TNF and IL-1). IL-6 in serum was assayed by using the IL-6-dependent cell line 7TD1 (IL-6). Sera from uninfected (uninf) mice were used as controls. The values are the mean ± the standard error of the mean of four animals per group in three independent experiments.
in all of the infected tissues were positive for IL-6 after 1 h. Like TNF and IL-1, tissue-specific cells, such as alveolar lining cells in the lung and sinusoid lining and Kupffer cells in the liver, expressed IL-6 protein (data not shown). Hepatocytes around the central vein in the liver were positive for IL-6 (Fig. 5, IL-6, liver) at 4 and 24 h. Lung tissue (48 h) exhibited extensive edema (Fig. 2, lung) and had strong, diffuse IL-6 reactivity (Fig. 5, IL-6, lung). In contrast to IL-1, intensive staining of IL-6 was noted in the vessels, including veins and arteries, in all tissues, particularly at 48 and 72 h (data not shown).

This observation is comparable to our in vitro model, in which expression of IL-6 by S. aureus-infected endothelial cells persisted for 72 h (36). The correlation of pathological changes at late time points in different tissues (discussed previously) with persistent high levels of IL-6 in tissues and sera suggests that IL-6 plays a contributory role in maintaining the systemic inflammatory response following activation of the proinflammatory cytokines TNF and IL-1.

**DISCUSSION**

*S. aureus* is a virulent pathogen that has the ability to cause a variety of potentially life-threatening infections. These infec-
tions vary from superficial soft tissue abscesses to septic shock (2, 32). A limited number of studies have examined the pathogenesis of gram-positive sepsis, in particular, the host response to these infections. Elevated levels of IFN-γ, IL-1, and TNF in serum have been observed during staphylococcal infection in previously reported in vivo studies (11, 16, 21, 31). However, the role of the cytokines in the pathogenesis of S. aureus infections has not been well characterized. The purpose of this study was to use an animal model of S. aureus bacteraemia to infect mice to correlate cytokine gene expression with bacteriologic and histopathologic findings.

Surprisingly, only a limited number of studies have examined the pathology of S. aureus sepsis in humans. A number of histopathologic observations from our study are similar to the reported findings on humans with invasive S. aureus disease. The leukocytic infiltration, focal pneumonitis, and edema found in mice are observed in patients with systemic S. aureus infections (14, 22, 33, 34). Myocardial abscesses are often found in cases of infective endocarditis (7). Metastatic seeding of other organs with abscess formation is a common complication of S. aureus bacteremia (22, 33). The pathological changes observed in the livers of infected mice, such as the presence of vacuoles and microabscesses, have also been observed in patients with bacterial sepsis. Our histopathologic data suggests that this animal model reproduces many of the pathological findings of S. aureus infections in humans and is therefore a useful model for further study of host-pathogen interactions in S. aureus-induced sepsis (14, 22, 33, 34). Other models, including those of cutaneous or prosthetic device infections, might be useful in studying the mechanisms of septic shock in humans. A number of studies of animals treated with LPS show increased levels of inflammatory cytokines in serum as well as tissue injury (8, 17, 19, 30). Although the inflammatory response after treatment with LPS is similar to the results of this study, the cause of cytokine expression and the contribution to the pathologic changes in our model are not known. CD14, the LPS receptor, is required for LPS function. Whether the same or a similar mechanism is involved in cytokine induction by S. aureus components is still under investigation (12).

The results of this study provide a baseline for subsequent studies on the role of inflammatory cytokines in the pathogenesis of staphylococcal diseases, including sepsis. The study demonstrates that following infection, bacteria are rapidly cleared from the blood, liver, and spleen. Despite this, there is continued cytokine expression and tissue damage. While it is possible that bacterial components, such as peptidoglycan, remain as stimuli, it is likely that the inflammatory process, once initiated by bacteria, is amplified by cytokines. This is the first animal model used to study in detail the correlation of cytokine gene expression with S. aureus infection. It does not establish a causal relationship between the expression of cytokines and the histopathologic changes noted. To clearly establish such a relationship, studies would need to be performed with specific anticytokine antibodies or mice with cytokine gene knockouts. Further study will focus on the cytokine gene expression induced by S. aureus components and prevention of some of the consequences of gram-positive sepsis by down regulation of inflammatory cytokine expression.

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