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Internalization of *Staphylococcus aureus* by Endothelial Cells Induces Cytokine Gene Expression

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The ability of the vascular endothelium to elaborate cytokines in response to gram-positive sepsis has received limited attention. This study examined cytokine expression by human umbilical vein endothelial cells (EC) following infection with a gram-positive bacterial pathogen, *Staphylococcus aureus*. *S. aureus* infection of EC resulted in the production of interleukin-6 (IL-6) and IL-1β. For IL-6, message was detected at 3 h after infection, protein was present at 24 h, and both message and protein persisted for 72 h. IL-1β message was detected at 12 h, IL-1β protein was detected at 24 h, and both persisted for 72 h. Message for colony-stimulating factor 1 remained unaltered. UV-killed *S. aureus* also elicited IL-1β and IL-6 message and protein expression at 24 and 48 h. Twenty-one clinical isolates of *S. aureus* were tested, and all induced IL-6 release by 48 h. However, the laboratory strain 8325-4 did not induce cytokine expression at any time point and was internalized by EC 1,000-fold less than other strains were. Internalization of latex beads by EC did not induce IL-6 gene expression. Furthermore, cytochalasin D treatment of the EC prevented IL-1 and IL-6 induction by *S. aureus* but not by tumor necrosis factor alpha or lipopolysaccharide. These results indicate that *S. aureus* is a potent inducer of IL-1 and IL-6 in EC and that internalization of *S. aureus* by EC is necessary for their cytokine expression. Thus, our data suggest that the vascular endothelium may play an important role in the pathogenesis of septicemia caused by gram-positive organisms.

The morbidity and mortality caused by infections with gram-positive organisms are comparable to those caused by infections with gram-negative organisms. Despite this, considerably less is known about the pathogenesis of the former disease, especially about the events that initiate sepsis in infections with gram-positive organisms or the role of the vascular endothelium in this process (5, 25). *Staphylococcus aureus* is a frequent cause of gram-positive sepsis and appears to have a unique tropism for endovascular tissue (14, 19, 23, 36). It was therefore selected for use in this investigation of the cytokine response of infected endothelial cells (EC).

The endothelium both initiates and responds to a cascade of events triggered by bacterial infection (2, 5, 20). The elaboration of cytokines by EC during sepsis initiates a series of events, including amplification of the cytokine response, that can be detrimental to the host. Numerous studies have focused on the cytokine response of EC to gram-negative organisms (5, 21, 25, 27, 28). The principal trigger of this response is lipopolysaccharide (LPS), or endotoxin, which induces the expression of interleukin-1 (IL-1) and IL-6 in EC. IL-1 and IL-6 are important mediators of the inflammatory response in vivo in sepsis caused by infections with gram-negative organisms (16). In humans, IL-1 and IL-6 levels correlated with disease severity (6–8, 12), and antibodies to these cytokines reduced mortality in experimental animal models of sepsis caused by infections with gram-negative organisms (11, 24, 34). The purpose of this study was to define the cytokine response of the vascular endothelium to *S. aureus* infection in vitro.

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MATERIALS AND METHODS

Preparation of bacteria. Blood culture isolates of *S. aureus* were obtained from patients with bacteremia or endocarditis. The standard strain used is the Wb strain, a clinical isolate used in previous studies, from a patient with endocarditis (3, 33). In addition, the laboratory isolates 8325-4 and a protein A deletion mutant of this strain, DU5723 (kindly provided by T. Foster, Dublin, Ireland), were used. Strain 8325-4 is a derivative of NCTC 8325, a human isolate, that has been cured of prophages (26). It is the standard laboratory strain used for staphylococcal genetic studies (22, 26). Bacteria were stored in nutrient broth (Difco Labs, Detroit, Mich.)–15% glycerol at −70°C and were subcultured onto blood agar. Fresh colonies were inoculated into Todd-Hewitt broth (BBL, Cockeysville, Md.) and grown overnight at 37°C. Two hours before the experiment, the overnight culture was resuspended in fresh Todd-Hewitt broth and grown into logarithmic-phase growth. Bacteria were collected by centrifugation, washed, and resuspended in medium 199 (M199; GIBCO, Grand Island, N.Y.). Bacterial concentration was measured spectrophotometrically at a wavelength of 620 nm, and the bacterial inoculum was confirmed by colony counts. For UV-treated *S. aureus*, an overnight culture was suspended in M199 and UV irradiated for 15 min as previously described (19). The bacteria were then washed and resuspended in M199 at the same concentration as the live strain. To ensure that the bacteria were killed, the organisms were plated in heart infusion agar (BBL).

EC preparation. EC were harvested from human umbilical veins with 0.1% collagenase (Sigma Chemical Co., St. Louis, Mo.) and were maintained in tissue culture at 37°C in 5.5% CO_2 as previously described (13, 23). Cells between the third and sixth passage were grown to confluence in 100-mm-diameter tissue culture plates (Falcon, Cockeysville, Md.) and passaged every 4 days in medium consisting of M199 supplemented with 20% newborn calf serum (GIBCO), 5% human AB serum, 1.6 mM glutamine (GIBCO), 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) buffer (Calbiochem-Behring, La Jolla, Calif.), 50 μg of porcine heparin (Sigma) per ml, 50 μg of ascorbate (Fisher, Fairlawn, N.J.) per ml, partially purified acidic fibroblast growth factor (10 μg/ml), penicillin, and streptomycin (10, 13).

Infection of EC. *S. aureus* was adjusted by optical density to an inoculum of 5×10^8 CFU/ml. EC (2×10^6 per 100-mm-diameter plate) were incubated with 10 ml of *S. aureus* for 30 min at 37°C. The plates were then washed twice with M199 and incubated with complete medium without antibiotics but with lysostaphin (10 μg/ml) (Applied Microbiology, New York, N.Y.) to lyse extracellular staphylococci. At different time points, supernatants were collected, filtered, and stored

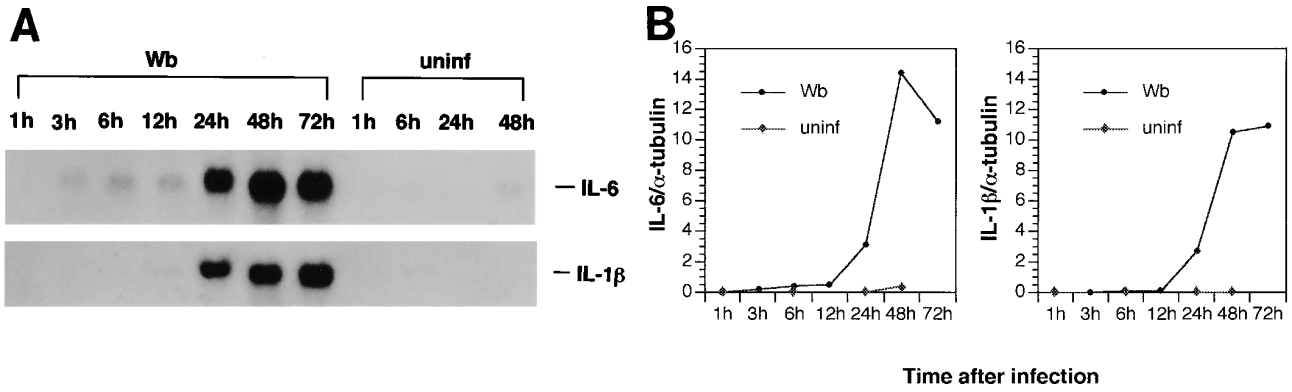

FIG. 1. (A) Northern blot analyses of IL-6 and IL-1β expression by *S. aureus*-infected EC. Total RNA was extracted from uninfected EC (uninf) or EC infected with *S. aureus* (Wb) for 30 min and then incubated in medium plus lysostaphin for 1, 3, 6, 12, 24, 48, and 72 h. RNA (15 μg) was separated on a 1.2% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled IL-6 or IL-1β. (B) Graphic representations of the ratios of IL-6 and IL-1β to the α-tubulin signal obtained from scanning the Northern blots illustrated in panel A.

at −20°C for cytokine protein determinations. The EC were harvested and collected for RNA extraction.

Infection assays. Infection assays were performed to determine the numbers of adherent and internalized bacteria as previously described (19, 23). Briefly, following a 30-min infection with *S. aureus*, the nonadherent bacteria were removed by washing twice with M199. The cells were lifted with trypsin, disrupted in distilled water, serially diluted, and plated in heart infusion agar. The number of colonies reflected the total number of adherent and internalized *S. aureus* bacteria. The number of internalized bacteria was determined by incubating comparable plates with lysostaphin for 20 min at 37°C before lifting the cells with trypsin. Assays for each time point were performed in triplicate, and all experiments were performed three to six times.

Northern (RNA) blot analysis. Total RNA was extracted with guanidinium–phenol-chloroform by the method of Chomczynski and Sacchi (9). Total RNA (15 μg) was electrophoresed through a 1.2% agarose–formaldehyde gel, transferred to a nitrocellulose filter (Schleicher and Schuell, Keene, N.H.), and hybridized with [^{32}P]dCTP-labeled cDNA probes (multiprime DNA labeling systems; Amersham, Arlington Heights, Ill.). cDNA for IL-1β was provided by R. Modlin, University of California at Los Angeles (37), and IL-6 was provided by T. Hirano (38). To ensure that equivalent amounts of RNA were applied to each lane, each blot was rehybridized with a cDNA for α-tubulin (a gift of N. Cowan, New York University Medical Center) (18), a structural protein whose expression we have shown does not vary with *S. aureus* infection. cDNA inserts were excised from their plasmids prior to use. RNA from the Wb strain of bacteria was prepared and assayed as described above to demonstrate that *S. aureus* did not express message that cross-reacts with the probes.

IL-6 and IL-1 protein assays. Supernatants from infected EC were assayed for IL-6 production by using the IL-6-dependent cell line 7TD1 (obtained from J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium). IL-1α and IL-β proteins from the supernatant or from cells lysed by three freeze-thaw cycles were assayed by immunoassay kits (R&D Systems, Minneapolis, Minn.).

Internalization of latex beads by EC. Latex beads (polystyrene, 2.5% solids, 1-μm diameter; Polysciences Inc., Warrington, Pa.) were washed with phosphate-buffered saline (PBS) three times and diluted with M199 to a final concentration of 0.05%. EC were incubated with 10 ml of the beads in medium at 37°C for 30 min. EC were washed twice with M199 and incubated with complete medium. Electron microscopy was performed at 24 h as previously described (19). Cells were harvested for RNA extraction at different time points.

RESULTS

Expression of IL-6 and IL-1β message by *S. aureus*-infected EC. EC infected with the Wb strain of *S. aureus* expressed both IL-6 and IL-1β message, while uninfected EC cultures did not. IL-6 message was detected as early as 3 h after infection, and its expression persisted for 72 h, the last time point assayed. The kinetics of IL-1β message expression were different from those for IL-6, in that IL-1β message was first detected at 12 h. IL-1β expression also persisted for 72 h postinfection. A Northern blot illustrating this expression is shown in Fig. 1A. Figure 1B is a graphic representation of the IL-6 and IL-1β expression normalized to that of α-tubulin. To achieve cytokine induction, an initial 6×10^6 to 8×10^6 bacteria per 100-mm-diameter plate of EC (2×10^6 cells) was required (data not shown). Maximal cytokine induction required approximately 5×10^9 bacteria per plate of EC.

Expression of IL-6 and IL-1β proteins by *S. aureus*-infected EC. IL-6 protein was first detected by bioassay 12 h after infection, and its levels increased in an exponential fashion until 48 h. Uninfected cultures assayed at the same time points produced low levels of IL-6 protein. Figure 2 shows a logarithmic transformation of IL-6 protein levels from the supernatants of infected and uninfected EC.

IL-1α and IL-1β protein, as assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) were detected in the supernatants of infected cultures at 4, 24, 48, and 72 h after infection (Fig. 3). Considerable activity was also detected in cell lysates prepared from infected cells and may include the activity of precursor forms of the proteins. IL-1 activity in uninfected cell cultures was minimal or undetected, and this background activity was subtracted from the experimental values. Similar data were obtained from six independent experiments.

CSF-1 expression by *S. aureus*-infected EC. To determine whether other cytokines are expressed by EC upon infection with *S. aureus*, the induction of colony stimulating factor-1 (CSF-1) was examined. EC were infected with the Wb strain,

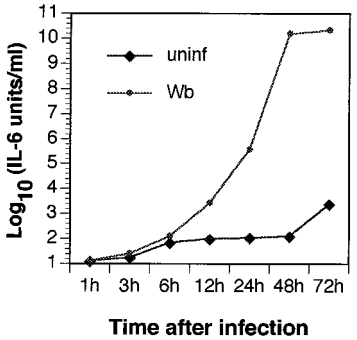

FIG. 2. Induction of IL-6 protein synthesis in EC infected with Wb. Supernatants were collected from EC cultures at different time points after infection and assayed for IL-6 protein by using the IL-6-dependent cell line 7TTD1. Supernatants from the uninfected EC (uninf) had no or very low levels of detectable IL-6 protein.

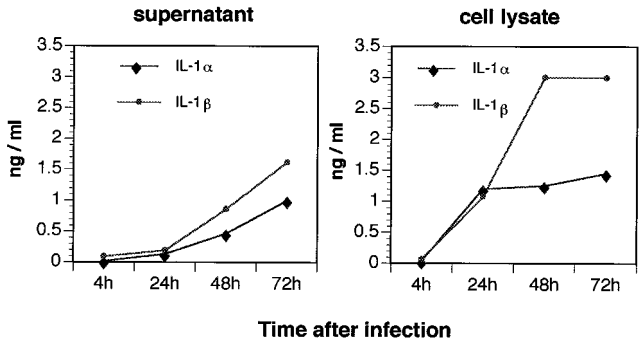

FIG. 3. ELISA to detect IL-1α and IL-1β in supernatants and cell lysates of Wb-infected EC. Supernatants were collected from EC cultures at different time points (4, 24, 48, and 72 h) after infection. Infected EC were then washed, harvested in PBS, and lysed by three freeze-thaw cycles.

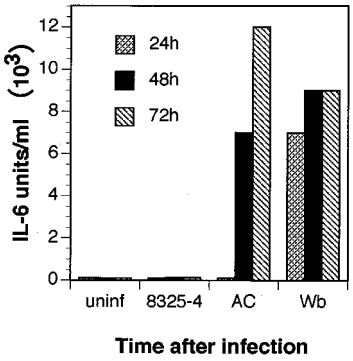

FIG. 5. IL-6 protein induction by three isolates of *S. aureus*. EC were infected for 30 min with Wb, 8325-4, or AC. Cell supernatants were analyzed for IL-6 protein at 24, 48, and 72 h after the initial infection period. Uninfected cells (uninf) and those infected with 8325-4 did not express IL-6.

and CSF-1 RNA was assayed 24, 48, and 72 h after the initial infection period. CSF-1 expression was not altered compared with that in uninfected cultures at any time point (data not shown).

UV-inactivated *S. aureus* elicits IL-6 expression by EC. We demonstrated previously that UV-killed *S. aureus* adheres to and is internalized by EC (19). To determine whether only viable bacteria were required for the induction of IL-6 or IL-1β message by EC, the Wb strain of *S. aureus* was UV irradiated and EC cultures were exposed to the killed bacteria. Message for IL-6 was detected 24 and 48 h after infection (Fig. 4) as well as message for IL-1β (data not shown). IL-6 and IL-1β proteins were also detected in the supernatants of experimental cultures (data not shown).

Induction of IL-6 expression in EC infected with different strains of *S. aureus*. To determine whether induction of IL-6 and IL-1 expression was unique to the Wb strain of *S. aureus*, 20 additional clinical isolates of *S. aureus* and the laboratory isolate 8325-4 were tested. EC cultures were infected with these strains and assayed for IL-6 message and protein 24, 48, and 72 h after the initial infection period. All of the clinical isolates tested induced IL-6 message and protein by 48 h postinfection. An example of the induction of IL-6 protein by one such clinical isolate, AC, in comparison with induction by Wb and the laboratory strain 8325-4 is shown in Fig. 5. AC induced IL-6 protein by 48 h after the initial infection period. There was considerable variability in the kinetics of this induction. Strain 8325-4 did not induce EC expression of IL-6 at any time point (Fig. 5), nor did it induce EC expression of IL-1β (data not shown). A derivative of 8325-4 (DU5723) also did not induce IL-6 or IL-1β expression (data not shown).

Internalization of different strains of *S. aureus* by EC. Internalization assays were performed to determine whether the various isolates of *S. aureus* assayed as described above were internalized differently. EC (2×10^5) were exposed to 6×10^8 to 14×10^8 CFU of each isolate of *S. aureus* per ml, and the number of internalized staphylococci was determined.

Strain 8325-4 was internalized 1,000-fold less than the other strains tested (Table 1). 8325-4 was the only strain that did not induce cytokine gene expression in EC. The number of 8325-4 bacteria adherent to EC did not differ significantly from that of the Wb strain.

Cytochalasin D inhibits the ability of *S. aureus* to induce cytokine expression by EC. To illustrate further the necessity of internalization of bacteria to induce cytokine gene expression, EC were treated with cytochalasin D, an inhibitor of endocytosis. The presence of cytochalasin D (1 μg/ml) in the media of EC cultures during *S. aureus* infection inhibited IL-6 gene expression (Fig. 6) as well as IL-1β gene expression (data not shown). Bacterial adherence to the EC surface was not affected by cytochalasin D but was not sufficient to induce IL-6. EC cultures treated with LPS (1 μg/ml) or tumor necrosis factor alpha (TNF) (200 U/ml) plus cytochalasin D were still induced to express IL-6 and IL-1β. Therefore, cytochalasin D did not affect LPS- or TNF-induced EC cytokine gene expression.

Internalization of latex beads by EC does not induce cytokine gene expression. EC were incubated with latex beads (1-μm diameter) in order to determine whether cytokine gene expression was induced by biologically inert particles similar in size to staphylococci. Internalization of latex beads by EC was visualized by electron microscopy (Fig. 7A). Internalization of the beads did not cause IL-6 gene expression in EC, while internalization of *S. aureus* did (Fig. 7B).

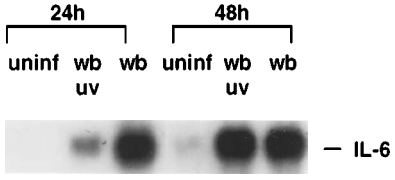

FIG. 4. EC infected with UV-killed Wb express IL-6 message at 24 and 48 h. EC were infected with UV-killed Wb (10^8 bacteria per ml) and assayed for IL-6 message. Live bacteria (Wb) were used as a positive control. uninf, uninfected.

TABLE 1. Adherence and internalization by EC of three strains of *S. aureus*[a]

Strain	Initial inoculum (10^8 CFU/ml)	No. of *S. aureus* cells/plate	
		Adherent (10^8)	Internalized (10^7)
Wb	6.3	1.0	5.8
AC	7.4	1.2	1.1
8325-4	14	0.9	0.002

[a] *S. aureus* bacteria were incubated with EC monolayers (in 100-mm-diameter plates) for 30 min, the surfaces of the cells were washed, and half of the cells were treated with lysostaphin (10 μg/ml) for 20 min at 37°C to lyse extracellular bacteria (number internalized). The bacteria from the other half of the cells were removed immediately (total number of bacteria). Aliquots from both sets were added to distilled water to rupture the cells and then were serially diluted and plated onto agar (19). The results are representative of five separate experiments.

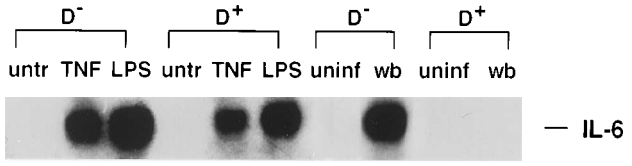

FIG. 6. Effect of cytochalasin D on IL-6 gene expression in EC. EC were incubated with cytochalasin D (1 μg/ml), an inhibitor of endocytosis, for 30 min before and during infection with Wb, or EC were treated with TNF (200 U/ml) or LPS (1 μg/ml). Total RNA from EC after 48 h of infection or from EC treated with TNF or LPS for 5 hours was assayed for IL-6 message. Cytochalasin D inhibited IL-6 gene induction in EC by the staphylococcal strain Wb but did not inhibit gene expression induced by TNF or LPS. D^+, with cytochalasin D; D^-, without cytochalasin D. uninf, uninfected.

DISCUSSION

Cytokine expression by human EC is recognized as one of the early events following infection by a variety of pathogens. EC not only are modified by circulating inflammatory mediators but also release a variety of cytokines that participate in the cascade of events that lead to septic shock (2, 5, 20, 21, 27, 28). Little is known about the role of the endothelium in the pathogenesis of gram-positive sepsis and, in particular, about the response of EC to infection with *S. aureus*. To address this issue, we developed an in vitro system to define the cytokine response of EC to *S. aureus* infection. The cytokines IL-6 and IL-1 were chosen because in human sepsis and in experimental animal models of bacterial sepsis (principally with gram-negative organisms), these cytokines are expressed (16, 35). Levels of IL-1 and IL-6 in blood are elevated in patients with sepsis and appear to correlate with clinical outcome (5, 6, 8, 12). TNF, another cytokine detected in the sera of patients with sepsis, is not produced by EC (1) and therefore was not examined in these studies.

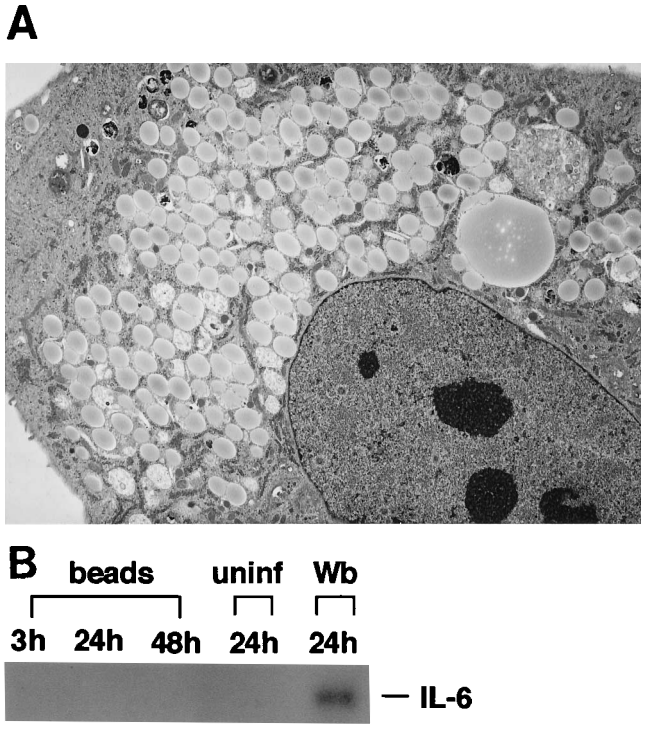

FIG. 7. (A) Transmission electron micrograph showing latex beads internalized by EC. (B) Northern blot analysis of IL-6 gene expression at different times following internalization of latex beads by EC. Total RNA from EC infected with live bacteria (Wb) was used as a positive control. uninf, uninfected.

Staphylococcal infection of cultured human EC induced IL-6 and IL-1β expression. Maximal induction occurred with a multiplicity of infection of 2×10^3 to 3×10^3 bacteria per EC in contrast with the three to four bacteria per EC necessary for minimal induction. On the basis of earlier (19) and unpublished observations, this correlates with approximately one to three internalized bacteria per EC for minimal induction and 50 to 100 internalized bacteria per EC for maximal induction. IL-6 was detected by 3 h postinfection, while IL-1β was not observed until 12 h. Both messages were persistent for 72 h after infection. This response is different from that observed with macrophages treated with LPS, in which case IL-1 is observed before IL-6 (15). The kinetics of IL-1 and IL-6 expression by EC following infection with different strains of *S. aureus* varied. Of note was the failure of the laboratory strain 8325-4 to induce either IL-1β or IL-6 over a 72-h period.

Binding and internalization studies demonstrated that 8325-4 bound to EC in a fashion comparable to that of the other clinical strains tested but was internalized at a much lower rate. The experiments using cytochalasin D demonstrated that bacterial adherence to the cell surface did not induce cytokine expression by EC and that when internalization of adherent bacteria was inhibited, no cytokine induction occurred. Cytokines were also not expressed when EC internalized latex beads. Thus, internalization appears to be necessary but not sufficient to induce expression of the cytokines IL-1 and IL-6. Also of interest was the observation that staphylococcal infection did not elicit a general cytokine response, since other factors such as CSF-1 were not affected.

Our studies suggest that one or more of the bacterial cellular components may be responsible for the cytokine response, since UV-killed bacteria were capable of inducing cytokine expression. The effect of UV-killed staphylococci on cytokine induction was delayed and suggests that bacterial replication and/or bacterial products may contribute to induction. The ability of bacterially secreted products, such as the enterotoxins, to induce cytokine expression in EC has not been examined. Studies with monocytes, however, have shown the induction of cytokines following exposure to staphylococcal peptidoglycan, toxic shock syndrome toxin 1, and lipoteichoic acids (4, 17, 30–32). Further studies are necessary to determine whether secreted staphylococcal products are also capable of stimulating EC.

The induction of IL-1β and IL-6 differed from the expression of Fc receptors by EC following *S. aureus* infection (reference 3 and unpublished observations). Fc receptors were expressed by EC 5 min following infection, suggesting that the proteins were already present in the cytosol and were released following perturbation of the cell. In contrast, the earliest detected expression of cytokines was 3 h after infection of EC, suggesting the need for new protein synthesis. Additionally, EC express Fc receptors upon exposure to and uptake of latex beads (29), whereas cytokine induction is not elicited by this process.

The EC response to infection may be central to the progression of septicemia. This study demonstrates that following *S. aureus* infection, EC produced IL-6 and IL-1. The nature and kinetics of this response suggest that the role of EC in cytokine expression may be distinct from the role of macrophages during infection. Further studies will focus on the induction mechanism and the bacterial components responsible for cytokine expression in *S. aureus*-infected EC, as well as on the role of the endothelium in cytokine expression in vivo.

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The first two authors contributed equally to this work.

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