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Recombinant Human Tumor Necrosis Factor Alpha Promotes Adherence of *Staphylococcus aureus* to Cultured Human Endothelial Cells

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Tumor necrosis factor (TNF), a potent inflammatory mediator secreted by monocytes during inflammation, was shown to significantly increase the adherence of *Staphylococcus aureus* to cultured human umbilical vein endothelial cells in vitro. The stimulatory effect of TNF was dose dependent and was bimodal with respect to time; bacterial adhesion peaked after 4 and 16 h of stimulation with recombinant human TNF-α. The ability of TNF-α to augment staphylococcal adherence to endothelial cells was contingent upon the presence of plasma factors. Thus, the complex interaction among cytokines (such as TNF), plasma factor(s), and the endothelium serves to modulate bacterial adherence to endothelial cells.

Septicemia due to *Staphylococcus aureus* is often the consequence of a local infection which has gained access to the bloodstream (13, 23). Once the bacteria enter the bloodstream, patients are at an increased risk of developing endocarditis (8, 24), pneumonia (7), and/or abscess as a result of metastatic complications (23). Acute infective endocarditis is a fulminant, life-threatening disease of the endocardium frequently caused by *S. aureus*. In contrast to the subacute form, acute infective endocarditis most commonly occurs in patients without underlying valvular abnormalities. The exact mechanisms by which staphylococci attach to the native endothelium are not well understood. Nonetheless, one can speculate that the bacterium must attach to the surface of the endothelium before invasion can occur.

Previous in vitro studies have demonstrated that *S. aureus* has an enhanced capacity to adhere to unstimulated endothelium compared with the majority of gram-negative bacilli (9, 15). Recent data from our laboratory have suggested that fibrinogen may be an important plasma mediator of staphylococcal adherence to unstimulated human endothelium (6). However, it is known that inflammatory and immunological processes in vivo are often associated with soluble mediators that are released by activated mononuclear phagocytes (1). Considerable evidence now supports the concept that immune and inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and gamma interferon can act directly upon endothelial cells (EC) to modulate endothelial function in pathologic processes. For instance, TNF causes EC to become markedly adhesive for polymorphonuclear leukocytes, monocytes, and lymphocytes (3, 19). Whether a cytokine such as TNF also modulates the adherence of *S. aureus* to EC is not known. In this study, we demonstrated that recombinant human TNF-α (rhTNF-α) promotes adherence of *S. aureus* to cultured human EC.

Two recent *S. aureus* human blood isolates from the Rockefeller University collection (strains LM and JA) and a *Staphylococcus epidermidis* strain (a gift from Dennis Mak of the University of Wisconsin at Madison) were used in the examination of bacterial adherence to TNF-stimulated EC. Because of the relative lack of protein A in strain LM (5), this strain is useful for examining protein A-independent adhesion to TNF-stimulated EC. *S. aureus* was maintained and grown in liquid chemically defined medium, while *S. epidermidis* was cultured in chemically defined medium supplemented with 10% Todd-Hewitt broth as previously described (6). Bacteria were radiolabeled in liquid culture by the addition of [*methyl*-^{3}H]thymidine (specific activity, ≈50 Ci/mmol; New England Nuclear, Boston, Mass.) to the medium (25 μCi/ml). The organisms were harvested, washed four times with phosphate-buffered saline (pH 7.4), resuspended in medium 199 (M199), filtered through a 5-μm filter to remove bacterial aggregates, and adjusted to an optical density of 1.0 at 650 nm (6). A subsequent Gram stain revealed the cell suspension to contain mostly single bacteria without cluster formation. CFU corresponding to an optical density of 1.0 at 650 nm (≈1.5×10^9 CFU/ml) were obtained by serial-tube dilution with subsequent plating on blood agar plates. The usual specific activities were approximately 1,000 cpm/2 × 10^6 cells for *S. aureus* strains and 1,000 cpm/1.5 × 10^6 cells for *S. epidermidis*, with 95% of the radioactivity being cell associated.

EC from human umbilical cords were obtained and cultured as previously described (10, 11). EC in the second or third passage were grown to confluence on gelatin-coated 96-well microtiter plates in the absence of antibiotics (6). Adherence of bacteria to EC was assayed essentially as previously described (6), with some modifications. Before the monolayer was exposed to tritiated bacteria, the medium (200 μl per well) in quadruplicate wells was aspirated and replaced at various timed intervals with equal volumes of growth medium (6, 10) containing various concentrations of rhTNF-α (Chiron Corp., Emeryville, Calif.). The control wells contained replaced medium alone. The effect of rhTNF-α on EC was verified by monitoring TNF-induced EC shape change as previously described (17, 18). Cell counting experiments indicated that the number of EC per TNF-treated well was the same as that of the untreated control. Following rhTNF-α exposure, the EC monolayers

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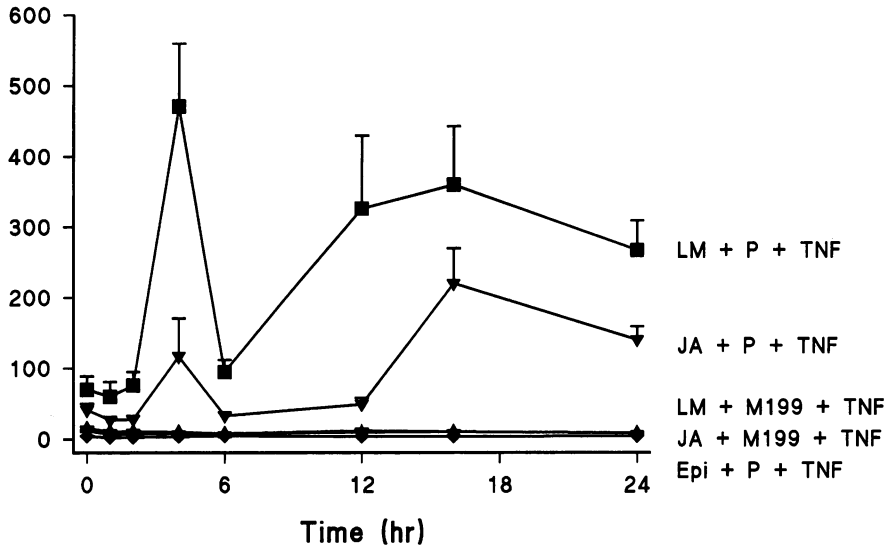

FIG. 1. Effect of time of preincubation of EC with rhTNF-α on staphylococcal adherence. Following preincubation with rhTNF-α at timed intervals, the monolayers were washed and incubated with either plasma (P) or M199. The adherence of *S. aureus* strains LM and JA or an *S. epidermidis* control (Epi) to EC was assayed as described in the text. The number of bacteria bound is shown as CFU per EC (mean ± standard error of the mean [SEM]). Experimental control values obtained with plasma or M199 alone with unstimulated EC are presented as datum points at 0 h. Adherence of *S. epidermidis* to rhTNF-α-stimulated EC in M199 (data not shown) did not differ from adherence to stimulated EC incubated with plasma. In points where SEM bars are not visible, the bars are smaller than the symbols.

were washed twice with M199 (200 μl each) to remove residual cytokine. To minimize EC detachment during the ensuing washing procedure, the monolayers were fixed lightly with 0.05% glutaraldehyde in M199 for 5 min and then washed twice with M199 (200 μl per wash) which also served as a quenching agent. This concentration of glutaraldehyde has previously been shown to be the minimal concentration necessary to anchor the EC monolayer to the underlying substratum and yet still retain the adherent characteristics of EC to the tested bacteria similar to that of nonfixed controls (6).

For the adherence assays, wells containing glutaraldehyde-fixed EC were preincubated with 35 μl of either M199 or heparinized plasma (6) diluted with an equal volume of M199 at 37°C for 30 min with agitation (200 rpm) and then washed twice with 200 μl of M199 each to remove excess proteins in plasma-treated wells. In some assays, serum obtained from the same plasma donor was used in place of plasma to coat EC prior to the addition of radiolabeled bacteria. In addition, M199 with heparin (10 U/ml) served as an additional negative control. Bacteria ($\approx 3 \times 10^7$ to 6×10^7 CFU in 35 μl) were then mixed with the monolayers and further incubated for 1 h with agitation. Following incubation, the monolayers were washed three times with 200 μl of M199 each. With this washing method, residual radioactivities associated with nonadherent bacteria in each well (e.g., *S. epidermidis* in M199) approached that of background. The EC and adherent bacteria were subsequently lysed with 200 μl of 2.5% sodium dodecyl sulfate–0.2 M NaOH (lysis buffer), and 100 μl of this lysate was neutralized with 400 μl of 0.05 M acetic acid and counted for radioactivity in the presence of scintillation fluid (Readysafe; Beckman Instruments, Fullerton, Calif.). The number of adherent bacteria in each well was derived from a standard curve of CFU versus radioactivity. With most studies, ≈1% of the labeled-cell inoculum showed nonspecific adherence to the tissue culture wells. Pilot studies also revealed that the presence of lysis buffer did not result in any appreciable degree of quenching.

To visually confirm staphylococcal adherence to EC, endothelial monolayers were grown to confluence in six-well tissue culture dishes as previously described (6). In some wells, the medium (550 μl each) in quadruplicate wells was aspirated and replaced at 4 h before the adherence assay with equal volumes of growth medium (6, 10) containing 10 ng of rhTNF-α per ml. The control wells contained replaced medium alone. After the EC were fixed with 0.05% glutaraldehyde, the wells were incubated with 550 μl of either M199 or plasma (diluted with an equal volume of M199) for 30 min at room temperature with agitation followed by two washes with 1.5 ml of M199 each. *S. aureus* bacteria and the *S. epidermidis* control (550 μl each) were added and further incubated for 1 h at room temperature with agitation. Following incubation, the wells were washed four times with M199, Gram stained, and examined under oil immersion (1,000×).

Two *S. aureus* strains (LM and JA) and an *S. epidermidis* control were used to examine the effect of various periods of preincubation of EC with rhTNF-α on bacterial adherence. In the presence of M199 alone, the addition of rhTNF-α (10 ng or 250 U/ml) to the growth medium at timed intervals (up to 24 h) did not increase the bacterial adherence of any of the three bacterial species tested compared with the adherence controls in the absence of rhTNF-α at 0 h (Fig. 1). In contrast, pretreatment of the monolayers with rhTNF-α in plasma-treated wells resulted in an appreciable increase in bacterial binding for both staphylococcal strains in comparison with that of nonstimulated plasma controls (i.e., at 0 h), whereas a similar effect was not seen with *S. epidermidis*. The adherence of *S. aureus* to plasma-treated EC which have been stimulated with rhTNF-α is time dependent. In particular, staphylococcal adherence to rhTNF-α-treated EC revealed a bimodal pattern of response for LM and JA

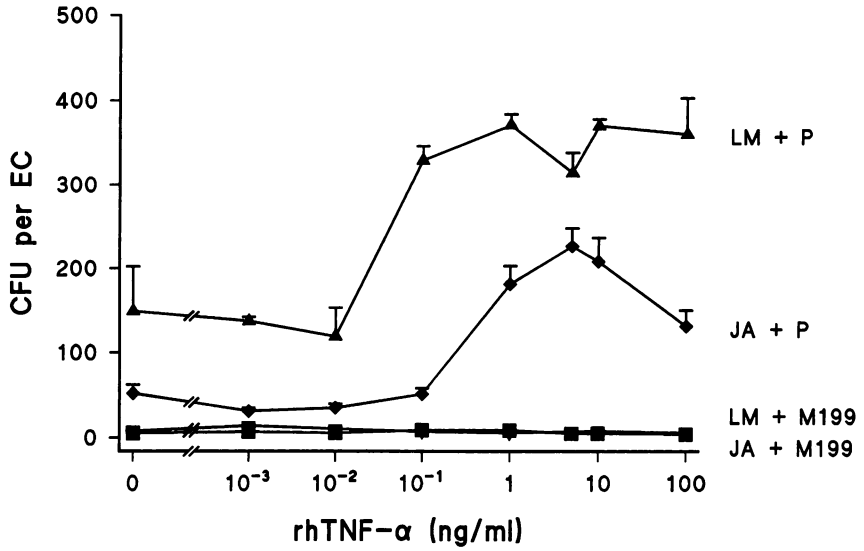

FIG. 2. Effect of rhTNF-α concentration on staphylococcal adherence to EC. EC were preincubated with various concentrations of rhTNF-α for 4 h before the bacterial adherence assay. The number of adherent bacteria was expressed as CFU bound per EC (mean ± standard error of the mean; $n = 4$).

(Fig. 1). Although both strains exhibited peak adherent activities to EC at 4 and 16 h of TNF stimulation, the magnitude of the response differed between the two strains tested. This augmentation effect at various timed intervals was not due to medium exchange in plasma-treated wells, since changing the growth medium at corresponding times without rhTNF-α potentiation did not result in any significant alteration of adherence pattern from that of nonstimulated controls at 0 h (data not shown). Since a 4-h incubation period with rhTNF-α markedly promoted bacterial adherence to EC, this duration of exposure to rhTNF-α was used in subsequent adherence assays.

The influence of rhTNF-α concentrations on staphylococcal adherence to plasma-treated EC was also studied. By using a range of concentrations (0.1 pg/ml to 100 ng/ml) to preincubate EC for 4 h, it was determined that the amplification in staphylococcal attachment to plasma-treated EC from rhTNF-α was dose dependent (Fig. 2). The stimulatory effect of rhTNF-α on staphylococcal adherence to EC was evident even at a concentration of 0.1 ng/ml. At concentrations between 5 and 10 ng/ml (125 to 250 U/ml), the potentiation effect of rhTNF-α appeared to be maximal. We have also found, as did others (14), that rhTNF-α was not toxic to EC within this concentration range, as determined by endothelial cell morphology and a lack of cell detachment from the underlying matrix (20). Studies by Pohlman and Harlan have also determined that human umbilical vein EC remained viable after exposure to rhTNF-α (20 ng/ml) for 18 h, as determined by ^{51}Cr activity retained in labeled EC monolayers (18). To maintain the experimental rhTNF-α level within clinical range and to avoid the possibility of endothelial damage from high doses of rhTNF-α, concentrations beyond 100 ng/ml were not tested.

Since it has been shown that rhTNF-α can alter EC morphology and consequently enlarge the junctional gap between cells (20), Gram stain studies were performed to ensure that the augmentation in staphylococcal adherence as a result of rhTNF-α stimulation was not due to increased bacterial binding to the junction gap. Results revealed that both LM and JA attached primarily to the surface of the EC (>90%) rather than to the junction gap between cells (data not shown). This finding is consistent with results recently reported by us in which visual confirmation of the enhancement in direct binding of *S. aureus* to EC in the presence of plasma factors was obtained (6).

To demonstrate the role of plasma factors in the enhancement of staphylococcal adherence to rhTNF-α-stimulated EC, both serum and plasma were used to coat EC before bacterial incubation. As displayed in Table 1, plasma was much more effective than serum in mediating bacterial attachment to rhTNF-α-stimulated EC. In addition, the augmentation effect of plasma was not due to heparin. As previously reported by us (6), these data confirm the finding that preincubation of the monolayer with plasma alone in the absence of rhTNF-α substantially increases the adherence of both LM and JA compared with M199 controls (Table 1).

TABLE 1. Effect of plasma and serum on staphylococcal adherence to TNF-stimulated EC[a]

Test solution	No. of staphylococci (CFU) bound per EC		
	LM strain	JA strain	*S. epidermidis* strain
M199 control	9.6 ± 1.3	7.6 ± 0.7	9.8 ± 1.2
M199 (stimulated)	9.2 ± 0.3	7.5 ± 1.0	7.7 ± 1.0
Serum	8.9 ± 0.6	14.0 ± 0.7	7.5 ± 1.3
Serum (stimulated)	7.7 ± 0.7	11.5 ± 0.2	5.6 ± 1.0
Plasma	134.0 ± 17.0[b]	52.0 ± 10.0[b]	8.0 ± 1.3
Plasma (stimulated)	293.0 ± 20.0[c]	208.0 ± 29.0[c]	7.7 ± 1.0
M199 + heparin	3.3 ± 0.4	5.6 ± 0.4	7.2 ± 2.7
M199 + heparin (stimulated)	2.8 ± 2.0	4.7 ± 0.3	6.2 ± 1.0

[a] EC were stimulated with rhTNF-α (10 ng/ml) in growth medium prior to the assay. Following light glutaraldehyde fixation (see text), the monolayers were incubated with different test solutions before radiolabeled bacteria were added. Values are expressed as mean CFU bound per EC ± standard error of the mean ($n = 4$).

[b] Statistically significant in comparison to the medium control (P value, <0.02 by t test).

[c] Statistically significant in comparison to plasma control (P value, <0.03 by t test).

Considerable evidence has accumulated to suggest that TNF-α is one of the central regulators of inflammation and immunity (1). The vascular endothelium, constituting a dynamic interface between blood and tissue, has been shown to respond to TNF by transiently expressing adhesion molecules (e.g., ELAM-1 and ICAM-1) for blood leukocytes (3, 17). These inducible functions associated with endothelial cell activation have been postulated to modulate inflammatory responses by altering the adhesion of lymphocytes and leukocytes to the endothelium in vivo (12).

Bacterial adhesion to native endothelium is one potential mechanism whereby bacteria migrate from the bloodstream into tissues during transient bacteremia (e.g., from catheters). However, the adhesive interaction between *S. aureus* and the endothelium is complicated by the presence of inflammatory mediators such as TNF-α that are frequently present during staphylococcal bacteremia. For this reason, we evaluated the attachment of *S. aureus* to confluent monolayers of human endothelium in the presence of rhTNF-α as an analog of the conditions found in vivo. The results of this study demonstrate that rhTNF-α can markedly increase the adherence of *S. aureus* to cultured human EC while *S. epidermidis*, an organism generally not known to cause acute intravascular infection on native endothelium, did not exhibit increased binding in the presence of rhTNF-α (Fig. 1). Although the magnitude of the adherence response differed for the two staphylococcal strains, the pattern of adherence to EC was similar. It is conceivable that the discrepancy in the magnitude of adherence may be ascribed to either strain variation or differences in the number of putative receptors to specific, as-yet-unidentified plasma cofactor(s). In addition, the kinetics of TNF-α in the promotion of staphylococcal attachment to EC revealed a bimodal pattern of response at 4 and 16 h after stimulation. Notably, a peak response to TNF-α at 4 h is similar to those found in the induction of leukocyte adhesion and procoagulant activity by TNF-α (16). This pattern of activation may suggest similar signaling pathways (1). Previous studies have also demonstrated that TNF, IL-1, and lipopolysaccharide act on EC and mediate the same or similar functions (e.g., procoagulant activity, ELAM-1 and ICAM-1 expression, and leukocyte adhesion [2–4, 17]). Likewise, TNF can induce EC to secrete IL-1 and vice versa (1). In addition, IL-1 has been found to enhance the adherence of *S. aureus* to human umbilical vein EC (21), while gamma interferon, another mediator that induces expression of some endothelial cell surface antigens (17), has no effect. Thus, the bimodal response pattern in bacterial adherence (Fig. 1) may be attributable to secondary induction of IL-1 secretion by EC following primary TNF stimulation.

More recently, Tompkins and colleagues (22) have isolated from unstimulated EC a 50-kDa membrane protein that appears to bind to *S. aureus* in the absence of plasma factors. In the case of rhTNF-α-stimulated endothelium, the results of this study indicated that plasma factors greatly augment staphylococcal attachment to EC. Previous in vitro studies by Thomas et al. (21) also demonstrated that the adherence of *S. aureus* to EC was increased by prior preincubation of the bacteria with "fresh" serum. Although fibrinogen and possibly another plasma factor(s) mediate the adherence of *S. aureus* to unstimulated EC in vitro (6), the mediatory role of these blood factors in bacterial adherence to TNF-stimulated EC in vivo is unknown.

In this report, we provided evidence that TNF-α could promote staphylococcal adherence to EC. However, the stimulatory effect needs plasma cofactors. Elucidation of the specific plasma cofactors and the corresponding receptors on *S. aureus* and EC will be important in further defining the interaction between *S. aureus* and TNF-stimulated endothelium in vivo.

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