

Contribution of Tumor Necrosis Factor to Host Defense against Staphylococci in a Guinea Pig Model of Foreign Body Infections

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The contribution of the cytokine tumor necrosis factor (cachectin; TNF) to host defenses against staphylococcal foreign body infections was studied *in vivo*. In tissue cages subcutaneously implanted into guinea pigs, progressive infection was initiated by a very low inoculum (100 cfu) of *Staphylococcus aureus* with a success rate of 100%, as is frequently encountered in related clinical situations. Locally injected autologous bacterial components derived from the cell wall of *S. aureus*, in particular peptidoglycan, were very active in raising TNF levels in tissue cage fluid and in preventing the development of infection by the 100% infective dose of the test strain. Furthermore, injection of murine recombinant TNF into tissue cages could substitute for the bacterial components in preventing experimental infection by *S. aureus*. The protective effect of TNF-eliciting bacterial components could be neutralized by anti-TNF antibodies. A local increase in TNF levels might improve host defenses against staphylococcal foreign body infections.

Staphylococcal foreign body infections are a major source of patient morbidity and implant failure. Not only strains of *Staphylococcus aureus* endowed with numerous virulence determinants but also species of coagulase-negative staphylococci previously considered nonpathogenic are recovered on infected orthopedic prostheses or cardiovascular devices [1, 2]. Animal models have confirmed the high susceptibility of artificial implants to low inocula of staphylococci [3–5], even of weakly virulent strains. These observations confirmed the ineffectiveness of local host defense mechanisms against microorganisms colonizing the implants [2–5].

In a previously developed guinea pig model of foreign body infection, we have shown that progressive infection was regularly produced by a very low inoculum (10^2 cfu) of *S. aureus* Wood 46 in subcutaneously implanted tissue cages [4]. In contrast, the same strain was unable to cause significant infection when injected subcutaneously or intraperitoneally at an inoculum as high as 10^8 cfu in the absence of tissue cages [2, 4]. Significant defects in the phagocytic and bactericidal functions [2, 4, 5] of polymorphonuclear leukocytes were observed in the infected tissue cage fluid, an inflammatory exudate elicited by the subcutaneous implant.

In preliminary observations [6], we have shown that locally injected bacterial factors obtained from a laboratory strain of *S. aureus* could prevent experimental infection by the same strain. A number of recent studies [7–20] have shown that host production of cytokines by either nonimmune cells (fibroblasts, endothelial cells) or cells of the immune system [7, 8] significantly contributes to host defense against various pathogens [9–11]. One of the most potent cytokines is tumor necrosis factor (cachectin; TNF); its production by mononuclear phagocytes [7, 12] is strongly stimulated by bacterial endotoxin (lipopolysaccharide [LPS]) from gram-negative organisms [7, 12, 13] or by either fixed or heat-killed staphylococcal whole cells [14, 18]. Whereas several studies have focused on the pathophysiologic effects of circulating levels of TNF or other cytokines during gram-negative and -positive [14] bacteremic infections, none has reported in detail how cytokine release might contribute to host defense against staphylococcal infections. Furthermore, the precise contribution of different cell wall or membrane components of staphylococci or other gram-positive bacteria [19, 20] as inducers of TNF release is not entirely elucidated.

We investigated whether injecting staphylococcal cell wall components into an experimental model of staphylococcal foreign body infection could elicit strong local production of TNF and determined the effects of this increased production of TNF on host susceptibility to experimental *S. aureus* infection.

Materials and Methods

Tissue Cage Implantation

Rigid perforated polytetrafluoroethylene (Teflon) tubes (internal and external diameters, 10 and 12 mm, respectively;

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length, 32 mm) were implanted subcutaneously into guinea pigs as previously described [4]. Animals were used for further experimentation 3–6 weeks after tissue cage implantation. An important characteristic of this model is that the perforations permit ingress of inflammatory cells into tissue cage fluid, in particular polymorphonuclear neutrophils and monocytes as previously described in detail [4].

Preparation of Soluble Bacterial Factors

Crude lysate. One liter of an overnight culture of *S. aureus* Wood 46 in Mueller-Hinton broth was washed and suspended in 10 ml of PBS (GIBCO, Paisley, Scotland). The concentrated suspension (10^{11} cells/ml) was then lysed for 2 h at 37°C with a mixture of lysostaphin (10 units/ml; Sigma, St. Louis), DNase (10 µg/ml; Sigma), and RNase (10 µg/ml, Sigma) by a modification of a previously published procedure [21]. Thereafter, the enzymes in the bacterial lysate were inactivated by heating at 90°C for 30 min [21]. The lysate was cleared from particulate material by two sequential centrifugations at 3000 g for 30 min and 100,000 g for 60 min, respectively. The protein content of the solubilized factors ranged from 250 to 520 µg/ml, as estimated by protein assay (Bio-Rad Laboratories, Munich). Each preparation was checked for complete heat inactivation of lysostaphin activity by showing the absence of lethal effect on exponentially growing test organisms. Accordingly, 10^6 cfu of *S. aureus* Wood 46 was incubated in vitro at 37°C for 60 min in a 1-ml mixture containing 400 µl of PBS, 250 µl of tissue cage fluid, and 250 µl of either solubilized bacterial factors or PBS as a control, and viable counts were made at 0 and 60 min.

Partially purified factors. The prophylactically active soluble factors were partially purified from the bacterial lysate by extraction with an equal volume of 10% trichloroacetic acid (TCA) for 2 h at 4°C. About 99% of the protein components of the bacterial lysate were precipitated by TCA and removed by low-speed centrifugation, as estimated by the Bio-Rad assay. Thereafter, the TCA-solubilized bacterial components were concentrated by overnight precipitation at 4°C with 5 vol of 100% ethanol followed by low-speed centrifugation. This ethanol precipitate was solubilized in PBS and dialyzed overnight at 4°C against PBS to remove traces of TCA, in membrane tubing (Spectrapor; Spectrum, Los Angeles) with a molecular weight cutoff of 12,000–14,000. Efficient removal of TCA was assessed by checking the pH of the final solution of partly purified bacterial factors; a value of 7.2 was usual.

Purified cell walls. Purified cell walls of *S. aureus* Wood 46 were prepared as described [22]. Briefly, after inactivation of autolytic enzymes by heat treatment (20 min, 100°C), cells were disrupted with glass beads and the cell debris separated by centrifugation for 20 min at 20,000 g. After washing once with distilled water, membrane residues were removed by treatment with 1% Triton X-100 for 30 min at 30°C, and the crude cell wall was thoroughly washed (four times) with distilled water. Crude cell wall was further depleted of contaminating proteins by extensive proteolytic digestion with 0.5 mg/ml trypsin for 16 h at 37°C in 0.05 M phosphate buffer at pH 7.8, washed five times with distilled water, and lyophilized. Quantitative analysis of the cell wall preparation was done after hydrolysis with 4 N

HCl for 16 h at 100°C and showed minimal contamination (<5%) by amino acids other than those normally found in peptidoglycan [22, 23]. Compared to glutamic acid, the molar ratios of major cell wall amino sugars and amino acids were 0.98 for muramic acid, 1.91 for glucosamine, 2.05 for alanine, 1.18 for lysine, 3.81 for glycine, and 0.16 for serine.

To solubilize prophylactically active components, we incubated a 1-mg/ml suspension of purified cell walls with lysostaphin, DNase, and RNase as above. After 2 h, the preparation was also heated for enzyme inactivation as described above, centrifuged at 100,000 g for 60 min to remove unsolubilized material, and stored at –20°C.

Cell wall teichoic acid and peptidoglycan. Ribitol teichoic acid was purified by extraction of undigested, purified cell walls (5 mg/ml) with 10% TCA for 72 h at 4°C, with occasional stirring [24]. After centrifugation at 20,000 g for 20 min at 4°C, the TCA-solubilized teichoic acid was concentrated by precipitation with 5 vol of cold ethanol. After centrifugation, the teichoic acid precipitate was redissolved in 10% TCA and reprecipitated with 5 vol of cold ethanol, then solubilized in water and lyophilized. The phosphate content of purified teichoic acid was 300 µg/mg as determined by a colorimetric assay [25].

The TCA-insoluble peptidoglycan component of cell walls was concentrated by centrifugation, then washed repeatedly with distilled water and lyophilized. Unlike purified teichoic acid, TCA-purified peptidoglycan had a residual phosphate content of only 30.6 µg/mg, which indicated a low level of contamination by teichoic acid incompletely extracted by TCA. Quantitative analysis of TCA-purified peptidoglycan, after hydrolysis with 4 N HCl for 16 h at 100°C, showed a shift in the molar ratio of glucosamine to glutamic acid from 1.98 before TCA extraction of cell walls to 0.93 after TCA extraction. In addition, TCA-purified peptidoglycan [25] contained molar ratios of alanine, glycine, lysine, and serine equivalent to those assayed in purified cell walls [26].

To test the biological properties of teichoic acid-free peptidoglycan, we prepared this purified component by an alternative procedure, namely by releasing ribitol teichoic acid from undigested, purified cell walls treated with 60% hydrofluoride (Riedel-de Haen, Seelze, Germany) as described previously [27, 28]. The phosphorus and ribitol content of peptidoglycan purified by this procedure was reduced to <5% compared with trypsinized cell walls. Consequently, glucosamine was present in the amount that corresponds to peptidoglycan. Analysis of the amino acids and amino sugars clearly indicated that the peptidoglycan was not changed by treatment with hydrofluoride.

Screening for Endotoxin and Exotoxin Contamination

Preparations of bacterial components were systematically screened to exclude significant endotoxin contamination, as assayed by the *Limulus* amoebocyte lysate test (sensitivity, 10 pg/ml; Associates of Cape Cod, Woods Hole, MA), and none contained detectable endotoxin. In addition, since recent studies also showed that a number of staphylococcal exotoxins could induce TNF production, we had to exclude their contribution in the preparations. The possible presence of staphylococcal enterotoxins was investigated by an ELISA (type A-B-C-D; Bommerli

Laboratories, Bern, Switzerland) according to the manufacturer. None of these enterotoxins was produced by *S. aureus* Wood 46 (limit of detection of the test, 0.1–1 ng/ml). Toxic shock syndrome toxin (TSST)-1 was determined serologically by gel diffusion [29]. Although TSST-1 production was demonstrated in supernatants of *S. aureus* Wood 46 cultures, this activity was consistently absent from purified cell walls or their purified components. Finally, the presence of staphylococcal α -toxin, of which *S. aureus* Wood 46 is a rich producer, was also screened for by a hemolytic assay against rabbit erythrocytes as previously described [30]. None of the preparations showed any significant hemolytic activity.

Experimental Infections and Protection Experiments

S. aureus Wood 46, a protein A-deficient strain, was used as the infecting microorganism for experimental tissue cage infection [4, 5]. Occasionally, *S. aureus* Cowan 1, a protein A-rich strain, or *Staphylococcus epidermidis* KH 11, a clinical isolate from a catheter-related sepsis [31], were used. Overnight cultures of bacteria in Mueller-Hinton broth were washed and resuspended in 0.9% NaCl. Tissue cages were inoculated with 0.2 ml of a 10^{-7} dilution, which usually contained $0.8\text{--}1.2 \times 10^2$ cfu/ml, as checked by plating on Mueller-Hinton agar.

The prophylactic administration of bacterial components was done by local inoculation of 0.5 ml (except when indicated) of the various preparations in 3 of 4 tissue cages per animal, at 2–3 h (except when indicated) before inoculation of live *S. aureus*. In addition, 1 tissue cage received 0.5 ml of sterile nonpyrogenic saline as a control. Frozen aliquots of enzymatically solubilized bacterial components, derived from either whole cell lysates or purified cell walls, were thawed just before use.

When purified ribitol teichoic acid or either TCA- or hydrofluoride-purified peptidoglycan were tested, they were not enzymatically solubilized. Instead, 1 mg from stored lyophilized preparations was freshly suspended in 1 ml of PBS by sonication for 15 s with a microsonicator (Branson sonifier 250, Danbury, CT) before being serially diluted for injection into tissue cages.

Experimental infection or protection was evaluated by quantitative bacterial cultures in aspirated tissue cage fluid (minimal detection level, 10 cfu) [4, 6]. Tissue cage fluid showing no growth (<10 cfu/ml) at 24 and 48 h after inoculation was scored as protected [6].

In Vivo Production of TNF

Routinely, a 0.2-ml sample of tissue cage fluid was aspirated to assay the baseline level of TNF just before prophylactic administration of the bacterial components (or control saline) into tissue cages. At 2 h, a second aspiration was done to assay the TNF level just before injection of *S. aureus* Wood 46. TNF levels were also evaluated after 24 h in tissue cages with culture-negative fluids. TNF activity in tissue cage fluid samples was measured using a cytolytic crystal violet assay with actinomycin D (1 $\mu\text{g}/\text{ml}$)-treated L929 cells (obtained from R. Ulevitch, Scripps Clinic, San Diego) as described previously [32]. Results were expressed in units per milliliter, 1 unit being defined as the reciprocal of the dilution leading to 50% lysis [32]. The reproduc-

Table 1. Prevention of staphylococcal tissue cage infection by local injection of soluble bacterial components (whole cell lysate) of *S. aureus* Wood 46.

Prophylactic substance	No. protected/ no. tested	Protection rate (%)
Saline	0/56	0
Whole cell lysate		
Untreated	26/26	100
Dialyzed	26/32	91
Partially purified components*		
Untreated	16/18	89
Trypsinized [†]	4/4	100
Digested with proteinase K [‡]	4/4	100

* Extracted with trichloroacetic acid and ethanol precipitated.

[†] Incubated with 100 $\mu\text{g}/\text{ml}$ trypsin for 30 min at 37°C, followed by addition of 200 $\mu\text{g}/\text{ml}$ trypsin inhibitor.

[‡] Incubated with 100 $\mu\text{g}/\text{ml}$ proteinase K, followed by heat inactivation of proteolytic enzyme for 30 min at 90°C.

ibility of the assay was assessed by testing the susceptibility to lysis of L929 cells by a standard curve of known concentrations of recombinant murine TNF (rTNF, obtained from B. Allet, Glaxo, Geneva) (specific activity, 10^7 units/ml). Basal levels in normal tissue cage fluid or serum were <30 units/ml, which was taken as the limit of specific TNF-induced cytolysis. The specificity of the cytolytic assay of guinea pig TNF was evaluated by the neutralizing effect of an anti-TNF mouse monoclonal antibody (MAb) (2A5 [IgG2a]; provided by D. Degroote, Medgenix, Fleurus, Belgium). Of many anti-human TNF MAbs, 2A5 was the only one that cross-reacted with guinea pig TNF, neutralizing 3–10 cytolytic units in the L929 assay at concentrations of 30–70 $\mu\text{g}/\text{ml}$.

Results

Prevention of experimental foreign body infection by bacterial factors from whole cell lysates. In the control group of guinea pig tissue cages injected with saline, 100% of cages ($n = 56$) inoculated with $0.8\text{--}1.2 \times 10^2$ cfu of *S. aureus* Wood 46 developed a permanent infection (table 1): Quantitative cultures of *S. aureus* in the tissue cage fluid yielded $10^4\text{--}10^7$ cfu/ml at either 1, 2, or 5 days after inoculation. In contrast, when 0.5 ml of soluble bacterial factors was injected into tissue cages 3 h before inoculation, infection was prevented in all the challenged cages ($n = 26$, table 1). Control experiments verified in vitro that the bacterial lysate per se contained no residual antibacterial activity, as could have resulted from incompletely inactivated lysostaphin. The test organisms (*S. aureus* Wood 46) incubated in vitro for 60 min at 37°C in PBS supplemented with 25% tissue cage fluid showed an increase in colony-forming unit counts of $97\% \pm 36\%$ (mean \pm SD, $n = 6$) in the presence of the soluble bacterial factors compared with $82\% \pm 35\%$ in their absence, thus

wall components, the most consistently active inducer of TNF production *in vivo* was TCA-purified peptidoglycan, which at 10 $\mu\text{g}/\text{ml}$ increased the median TNF titers from <30 to 5250 units/ml (range, 470–15,000; $n = 6$) in tissue cage fluid within 2 h (figure 1). Similar results were obtained with the teichoic acid-free hydrofluoride-purified peptidoglycan (data not shown). This increase in TNF titers was transitory, since values recorded at 24 h were <10% of the 2-h values (not shown).

Prevention of tissue cage infection by purified murine rTNF. Further experiments were done to evaluate whether purified murine rTNF could substitute for the solubilized bacterial factors in preventing tissue cage infection by *S. aureus*. Initial experiments to determine the optimal quantity and time lag of a prophylactic dose of murine rTNF in tissue cages indicated that 6800 units of the purified cytokine injected 30 min before inoculation of 10^2 cfu of *S. aureus* Wood 46 gave optimal results; in contrast, higher doses (13,400 and 28,600 units) were poorly effective and possibly toxic, as suggested by the hemorrhagic aspect of tissue cage fluids. Experiments in a total of 6 animals confirmed the good prophylactic effect of 6800 units of murine rTNF, which prevented tissue cage infection in 73% of the tissue cages ($n = 11$) challenged. Because 3400 units was clearly less effective, protecting only 33% of 6 tissue cages, this regimen was not further tested. Control experiments verified that murine rTNF incubated *in vitro* with *S. aureus* Wood 46 exerted no effect on growth or viability of the test strain (data not shown).

Inhibition of bacterial factor-induced protection by anti-TNF antibody. To further confirm that protection from experimental foreign body infection by cell wall components was mediated by an increase in TNF in tissue cage fluid, we tried to neutralize this cytokine activity *in vivo* by anti-TNF antibodies. A dose of 140 μg of anti-TNF MAb 2A5 (or isotype-matched MAb without neutralizing activity as a control) in 0.4 ml of PBS was injected into tissue cages 10 min before injection of a protective dose of peptidoglycan. Treatment with 2A5 MAb, but not with control MAb, significantly blocked in 6 of 8 tissue cages the peptidoglycan-induced protection against experimental infection with *S. aureus* Wood 46 ($P < .03$, Fisher's exact test with Yates's correction). Eight control tissue cages injected in parallel with 10 $\mu\text{g}/\text{ml}$ peptidoglycan alone were all protected from experimental infection with *S. aureus*.

Discussion

In this report, we showed that staphylococcal cell wall components consistently prevented locally induced staphylococcal experimental foreign body infection and that a significant host response, probably mediated by TNF (cachectin), was involved in such a prophylactic effect. The relationship between enhanced resistance to infectious challenge and lo-

cal increase in TNF concentrations was established as follows: TNF titers increased rapidly (within 2 h) and significantly after local injection of the prophylactic bacterial factors, injection of purified murine rTNF could substitute for the prophylactic effect of the bacterial factors, and anti-TNF antibodies in the tissue cage fluid were antagonistic of the prophylactic effect of bacterial factors.

Our preparations of prophylactically active staphylococcal components were screened either by the *Limulus* assay or by immunologic and functional assays for the presence of contaminating endotoxin or staphylococcal exotoxins, respectively. It was essential to exclude a significant contribution of these products to the prophylactic effect observed in our model, because endotoxin [7–13] and several staphylococcal exotoxins [33–35] are potent *in vitro* and *in vivo* inducers of TNF production and release. Although the staphylococcal strain used to prepare prophylactic components was an active producer of both α -toxin [30] and TSST-1 (unpublished data), neither of these exotoxins nor endotoxin could be demonstrated in our preparations of prophylactically active components. We assume that staphylococcal exotoxins were extensively removed from our preparations by the heating and chemical procedures used.

Attempts to explore at a cellular level by which mechanism(s) cell wall components of *S. aureus* induced TNF production in the tissue cage fluid have met with limited success. *In vitro* incubation of the most active prophylactic components with endotoxin-sensitive murine peritoneal macrophages did not show any significant TNF bioactivity (L929 assay), but small amounts of immunoreactive TNF were found (unpublished data). A possible discrepancy between TNF levels *in vivo* and the undetectable to low amounts of TNF produced by peritoneal macrophages stimulated with staphylococcal cell wall components could be derived from the results reported by Burchett et al. [36]. TNF production by freshly isolated human monocytes is readily stimulated by LPS. In contrast, the secretion of TNF in response to LPS of tissue macrophages was only moderate in the absence of interferon- γ . This suggests that the presence of other cells of the immune system, such as T cells, as producers of IFN- γ may be crucial to elicit the local response in the *in vivo* model.

Additional support for the concept of tissue-specific responsiveness of monocytes/macrophages comes from recent studies by C. P. Timmerman and A. Fleer (Utrecht, Netherlands, personal communication) who demonstrated a significant *in vitro* production of TNF by freshly isolated human monocytes incubated with purified peptidoglycan. In contrast, other studies have shown the absence of any significant *in vitro* TNF production by macrophages incubated with either pneumococcal cell surface components [19] or streptococcal peptidoglycan [20]. Finally, other recent reports suggest that lipoteichoic acid from either *S. epidermidis* [14] or from streptococci [20] may play a significant role in TNF

production by mononuclear phagocytes. Therefore, it appears likely that the presence of macrophages, fibroblasts, lymphocytes, and polymorphonuclear leukocytes could contribute to local TNF production [7]. A possible target of increased TNF production in tissue cage fluid might well be polymorphonuclear leukocytes, whose bactericidal activity was recently shown to be significantly activated by cytokines released from macrophages and lymphocytes [37].

In conclusion, our experimental model was a useful approach with which to study localized production of TNF and its potential role in host defense against staphylococcal infections. Further studies should focus on how TNF interacts with other components of the cytokine network and with phagocytic cells *in vivo* and *in vitro* to better understand defective host defense mechanisms against staphylococcal foreign body infections.

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