INFECTION AND IMMUNITY, Oct. 1996, p. 4351–4355 Vol. 64, No. 10
0019-9567/96/$04.00+0

Supernatants from *Staphylococcus epidermidis* Grown in the Presence of Different Antibiotics Induce Differential Release of Tumor Necrosis Factor Alpha from Human Monocytes

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Received 8 April 1996/Returned for modification 20 May 1996/Accepted 18 July 1996

Bacterial products from gram-positive bacteria, such as peptidoglycan, teichoic acid, and toxins, activate mononuclear cells to produce tumor necrosis factor alpha (TNF). The present study evaluated the release of soluble cell wall components from *Staphylococcus epidermidis* capable of inducing TNF after exposure of the bacteria to various antibiotics. A clinical *S. epidermidis* isolate (694) was incubated with either penicillin, oxacillin, vancomycin, or clindamycin at five times the MIC. Supernatants of the cultures obtained by filtration were added to plastic adherent monocytes in the absence or presence of human serum. After 18 h of incubation, monocyte supernatants were tested for the presence of TNF by enzyme-linked immunosorbent assay (ELISA). Supernatants from bacteria incubated with β-lactam antibiotics induced higher TNF levels than those obtained from bacteria incubated with culture medium only (no antibiotics), vancomycin, or clindamycin. Human serum potentiated supernatant-induced TNF release, especially in β-lactam supernatants. The soluble peptidoglycan and teichoic acid contents of supernatants, as estimated by inhibition ELISA and, for peptidoglycan, also by affinity depletion with vancomycin-Sepharose gel, were proportional to TNF release. Differences in the ability of individual antibiotics to generate TNF-releasing products from *S. epidermidis* were observed, the most potent antibiotics being penicillin and oxacillin.

Tumor necrosis factor alpha (TNF) is a cytokine that activates a cascade of secondary inflammatory mediators, which can eventually lead to endothelial-cell damage as well as to hemodynamic and metabolic derangements (3). TNF is regarded as a key mediator of both gram-negative and gram-positive bacterial septic shock (30). However, TNF also plays an important protective role, as judged from experiments with TNF knockout mice infected with pathogens such as *Listeria monocytogenes* and from animal studies dealing with foreign-body infection caused by *Staphylococcus aureus* (23, 31). Although *Staphylococcus epidermidis* is usually regarded as a commensal, it can cause sepsis and septic shock (5, 16, 24). During the last decade, the incidence of *S. epidermidis*-induced sepsis has increased dramatically. *S. epidermidis* infections are strongly associated with the medical use of foreign materials and are most often seen in immunocompromised patients (8, 16). Cell walls, peptidoglycan (PG), teichoic acid (TA), lipoteichoic acid, and toxins from staphylococci have all been shown to induce TNF and interleukin-1 release from human monocytes (1, 2, 10, 12, 14, 17, 19, 21, 32). Exposure of staphylococci to β-lactam antibiotics results in the release of soluble peptidoglycan (sPG) molecules, TA, lipoteichoic acid and alpha-toxin (6, 10, 14, 27, 36, 37). sPG molecules are non-cross-linked PG polymers that give rise to TNF and interleukin-1 release from human monocytes (10, 26, 34). Clindamycin, vancomycin, and β-lactam antibiotics are generally used in the treatment of staphylococcal infections. β-Lactam antibiotics exert their antibacterial effects by blocking the incorporation of newly synthesized sPG into the bacterial cell wall (37). Vancomycin is known to bind the D-alanine–D-alanine determinant found in non-cross-linked PG (7); this prevents sPG from being incorporated into the cell wall. Clindamycin inhibits protein synthesis at the ribosomal level (33). Earlier studies on *Escherichia coli* have demonstrated that the release of lipopolysaccharide (LPS) from the gram-negative cell wall and subsequent TNF induction varied with the antibiotic used (15, 25, 29). The present study investigated the effects of the different antibiotics mentioned on the release of bacterial products with TNF-inducing capacity from *S. epidermidis.*

Materials and methods. A clinical *S. epidermidis* isolate (strain 694), obtained from a patient with catheter-related bacteremia, was used for the generation of bacterial supernatants. Stock solutions of penicillin G (Sigma, St. Louis, Mo.) oxacillin (Sigma), vancomycin hydrochloride (Eli Lilly, Nieuwegein, The Netherlands) and clindamycin hydrochloride (Upjohn, Puurs, Belgium) in 10 mM phosphate-buffered saline (PBS; pH 7.4) were prepared and stored at −70°C until use. MICs for *S. epidermidis* 694 were determined by standard dilution techniques. The MICs obtained were as follows: penicillin, 2 μg/ml; oxacillin, 2 μg/ml; clindamycin, 0.5 μg/ml; and vancomycin, 1 μg/ml. *S. epidermidis* 694 was subcultured on blood agar. One colony was suspended in 5 ml of Mueller-Hinton broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) and incubated at 37°C overnight. The next day, the suspension was diluted 70-fold in prewarmed medium 199 (M199; Gibco BRL, Life Technologies, Paisley, Scotland) and incubated at 37°C until log phase, as determined by optical density in a turbidity photometer LPI (Lange, Berlin, Germany). The bacterial suspension was divided into 50-ml plastic tubes (Greiner Labortechnic, Nürtingen, Germany), with 8 ml in each tube, and 500-μl samples of antibiotic solutions were added to final concentrations of five times the MIC. Bacteria without antibiotic served as controls. The tubes were subsequently incubated at 37°C on a shaker. Bacterial suspensions were collected after 1-, 2-, and 4-h incubations and filtered (0.2-μm-pore-size filter;

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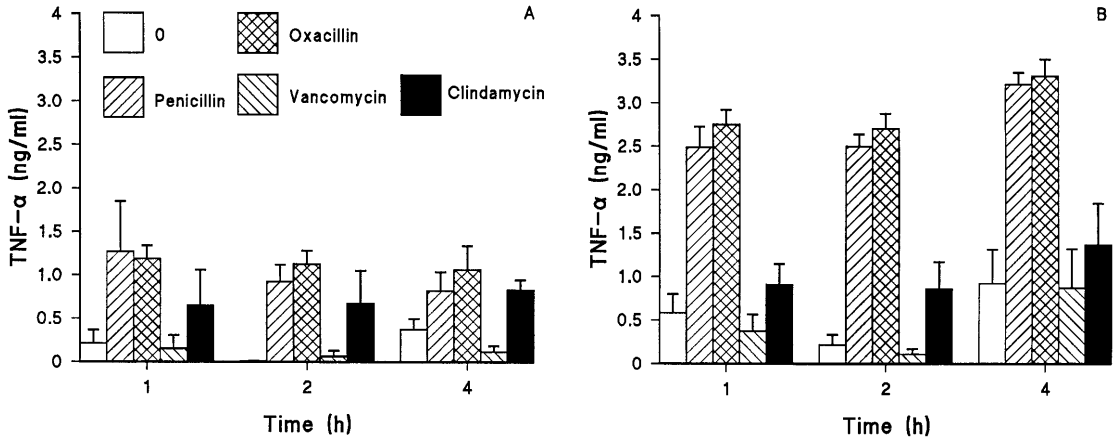

FIG. 1. TNF levels measured by ELISA in supernatants of human adherent monocytes stimulated with supernatants from antibiotic-incubated *S. epidermidis* in the absence (A) or presence (B) of 10% HPS. Supernatants were collected after 1, 2, and 4 h of incubation. Controls, including M199 and the antibiotics, did not induce TNF levels of >62 pg/ml when tested either in the absence or presence of 10% HPS (data not shown). Data represent the means plus standard errors of the means from three different experiments with monocytes from different donors.

Schleicher & Schuell GmbH, Dassel, Germany). The supernatants were then stored at −70°C until use.

Human peripheral blood mononuclear cells were isolated from buffy coats of healthy donors. Centrifugation on a Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) density gradient was performed as described by Böyum (4). Monocytes (10^6/ml) were suspended in M199 enriched with Earle's salt solution without serum or antibiotics. The cells were transferred to 96-well flat tissue culture plates (Nunc, Kamstrup, Denmark) and then incubated in 5% CO_2 at 37°C for 1 h to allow adherence. Nonadherent cells were removed by aspiration, and the monolayer of monocytes (examined by trypan blue staining and May-Grunwald and Giemsa staining and found to be >98% viable and >94% monocytes) was then stimulated with bacterial filtrates in the absence or presence of 10% human pooled serum (HPS) composed of 30 different healthy-donor sera. Some experiments included 5 μg of polymyxin B (PMB; Sigma) per ml to exclude any effects of endotoxin contamination. After incubation of the monocytes in 5% CO_2 at 37°C for 18 h, the supernatants were collected and centrifuged (10,000 × *g*, 5 min) to remove cells. Supernatants were stored at −70°C prior to TNF measurement.

TNF levels in supernatants were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) developed at our institute as described before (18). The minimum detection level of the test varied between 32 and 64 pg/ml. In the concentrations used, the antibiotics did not interfere with TNF detection.

The PG and TA contents of supernatants were estimated by inhibition ELISA (11). Microtiter plates (Costar) were coated overnight with PG (5 μg/ml) or TA (10 μg/ml) in saline at 37°C. PG and TA were purified from *S. epidermidis* 354 as described before, the purity being confirmed by estimations of phosphorus content and by amino acid, muramic acid, and fatty acid analyses, and endotoxin contamination being excluded by *Limulus* amoebocyte lysate assay (19, 22). Microtiter plates (Costar) were incubated with 1% gelatin (Merck, Darmstadt, Germany) in PBS–0.1% Tween 20 for 1 h to prevent nonspecific binding. Then the supernatants and standards, purified PG or TA, were diluted in PBS-Tween, and HPS was added to a final concentration of 1/1,000. These mixtures were incubated at 37°C for 1 h and then transferred to PG- or TA-coated plates and blocked (37°C, 1 h) with 1% gelatin in PBS-Tween. After a 1-h incubation at 37°C, peroxidase-labeled sheep anti-human immunoglobulin G (ICN Biochemicals, Amsterdam, The Netherlands) diluted in 2% sheep serum (Veterinary Faculty, Utrecht University, Utrecht, The Netherlands) was added and the mixture was incubated at 37°C for 1 h. Finally, substrate composed of tetramethylbenzidine and urea peroxide in 0.1 M sodium acetate buffer was added and allowed to be converted. After 10 min, the reaction was stopped by 2 N H_2SO_4 addition and the optical density was read with a microplate reader operating at 450 nm. The PG and TA contents of supernatants were estimated by comparing optical density values of supernatants with those of purified components. Comparisons were made at the 50% inhibition points. The antibiotics used did not interfere with PG or TA detection. PMB (50 mg) or vancomycin hydrochloride (80 mg) dissolved in 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl was added to washed 1.5-g portions of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions (7, 13). The gels were suspended in PBS. After 1 ml of gel had been allowed to settle and PBS had been removed by aspiration, 500 μl of supernatant was added. The gel-filtrate mixtures were then vortexed for 30 min. After resedimentation of the gel, supernatant obtained by centrifugation in a centrifuge filter unit (pore size, 0.22 μm; Costar) was added to monocytes with or without HPS (final concentration, 10%). LPS from *E. coli* O111:B4 (Sigma) together with 10% HPS served as a reference.

Results and discussion. Incubation of *S. epidermidis* with penicillin or oxacillin resulted in supernatants with the highest TNF-inducing capacity (Fig. 1). Clindamycin supernatants induced slightly lower TNF levels than β-lactam antibiotic supernatants, while supernatants from vancomycin-incubated bacteria or from bacteria alone induced the lowest TNF release. No enhancement of TNF levels was observed when bacteria and antibiotic were incubated longer than 1 h (Fig. 1). The antibiotics themselves did not induce TNF release. To exclude the possibility that any endotoxin contamination had occurred during the preparation of supernatants or monocyte stimulation, the 4-h incubation supernatants were tested in the absence or presence of PMB (Table 1). No differences in TNF release were observed according to whether supernatants were preincubated with or without PMB. We have shown before that PMB at the concentration used inhibits almost totally the

TABLE 1. TNF release by human monocytes induced by antibiotic-incubated *S. epidermidis* supernatants in the absence or presence of PMB

Antibiotic in culture	TNF concn (ng/ml)[a]	
	Without PMB	With PMB
Penicillin	0.930 ± 0.146	0.939 ± 0.159
Oxacillin	1.099 ± 0.253	1.019 ± 0.195
Vancomycin	0.119 ± 0.07	0.117 ± 0.01
Clindamycin	0.864 ± 0.104	0.734 ± 0.162
None (control)	0.425 ± 0.08	0.355 ± 0.257

[a] Values are means ± standard errors of the means.

TABLE 2. TNF-inducing activity of supernatants of *S. epidermidis*[a]

Antibiotic in culture	Expt	TNF concn (pg/ml)	
		PMB-Sepharose gel	Vancomycin-Sepharose gel
Penicillin	1	281	51
	2	497	175
Oxacillin	2	560	218
	3	794	120
Penicillin + 10% HPS	1	1,661	471
	2	2,107	1,063
	3	2,530	1,635
None (control)[b]	1	145	1,530
	2	<32	2,676
	3	375	3,225

[a] Penicillin supernatants in the presence or absence of 10% HPS and oxacillin supernatants only in the absence of HPS were incubated with human monocytes after affinity depletion of the supernatants with PMB (LPS binder)- or vancomycin (sPG binder)-Sepharose gel. LPS (100 ng/ml) was used as a positive reference for the PMB-Sepharose gel.
[b] 100 ng of LPS per ml + 10% HPS.

TNF release induced by LPS (100 ng/ml) from *E. coli* O111:B4 (19). The supernatants used to generate Fig. 1A were also preincubated for 10 min with 10% HPS before the mixtures were added to the cells. HPS enhanced the TNF release of all supernatants (Fig. 1B). In general, the serum-induced increase of TNF secretion varied from two- to fourfold. Penicillin and oxacillin supernatants induced higher TNF levels than clindamycin, vancomycin, or control supernatants.

Since HPS contains antibodies against PG and TA (6, 28, 35), it was used as a detecting agent in inhibition ELISAs that were based on the principle that sPG and released TA interfere with the binding of HPS antibodies to solid-phase-coated, purified PG and TA. The β-lactam antibiotics penicillin and oxacillin gave rise to equally high levels of sPG (Fig. 2A), estimated at 50% inhibition. This level was about 10-fold higher than that in supernatants without antibiotics or with vancomycin or clindamycin. TA levels were also more enhanced in β-lactam supernatants, being about fivefold higher than those in clindamycin, vancomycin, or control supernatants (Fig. 2B). Representative standard curves of cell wall PG (0.5 mg/ml undiluted) and TA (1 mg/ml undiluted) are shown in Fig. 2. Since vancomycin binds selectively to D-Ala–D-Ala residues on sPG and since PMB neutralizes LPS but not sPG, vancomycin and PMB were coupled to Sepharose gels to reduce sPG and LPS levels by affinity absorption. After the passage of supernatants over Sepharose columns, relatively low TNF levels were induced (Table 2 compared with Fig. 1 and Table 1). Preincubation of penicillin and oxacillin supernatants with vancomycin-Sepharose gel decreased the TNF levels by 52 to 74%, depending on whether HPS was present in the monocyte stimulation test (Table 2). In contrast, an LPS solution containing 100 ng of *E. coli* O111:B4 per ml in M199 was selectively depleted by PMB-Sepharose gel, resulting in a decrease in TNF levels of up to 93% (Table 2). To exclude interference by heat-labile exotoxins, supernatants were heated at 60°C for 1 h or at 100°C for 15 min before testing. No decrease in the TNF-inducing capacity of the supernatants was observed upon heating. Delta-toxin, which is heat stable, was quantitated by hemolytic assay (9). Although the toxin was detectable in all supernatants, toxin levels did not show any correlation with TNF secretion (data not shown).

This in vitro study shows differences between antibiotics with regard to their ability to generate bacterial products from *S. epidermidis*, which in turn induce TNF release from human monocytes. Supernatants of penicillin- and oxacillin-incubated

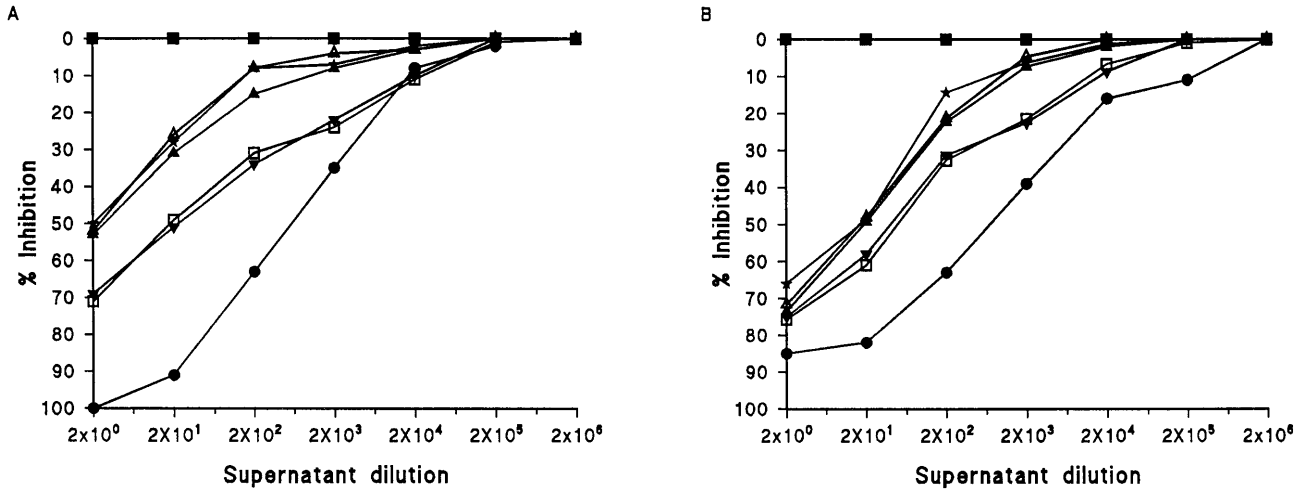

FIG. 2. Dose-dependent inhibition of antibody binding to purified, solid-phase-coated PG (A) and TA (B) by culture supernatants of *S. epidermidis* obtained in the presence or absence of different antibiotics. M199 was the control medium. Data are expressed as the means of three different 1-h incubation experiments. Symbols: ■, M199; ▲, no antibiotic; ▼, penicillin; □, oxacillin; △, vancomycin; ✶, clindamycin; ●, PG reference (0.5 mg/ml) (A) or TA reference (1.0 mg/ml) (B).

S. epidermidis induced substantially greater TNF release than supernatants from vancomycin- or clindamycin-treated bacteria or from bacteria not treated with antibiotics, especially in the presence of serum. A slightly enhanced TNF release in supernatants from vancomycin-incubated, but not clindamycin-incubated, *S. epidermidis* was obtained when 20 times the MIC instead of 5 times the MIC was used. However, TNF levels did not exceed the clindamycin-induced TNF levels (data not shown).

Earlier studies have shown that sPG and TA are released during β-lactam treatment of staphylococci and can induce TNF release by human monocytes (19, 26, 34, 37). This makes sPG and TA probable candidates for the enhanced TNF secretion induced by β-lactam supernatants. Although we did not look at other surface components, we consider sPG and TA major TNF-inducing components of β-lactam supernatants but not of vancomycin supernatants. This is in agreement with studies by Zeiger et al. (37) and Mirelman et al. (20), who showed that clinical isolates of *S. aureus* and *Micrococcus luteus* were capable of secreting sPG when they were grown in medium containing penicillin G but not in medium containing vancomycin. In another study, Zeiger et al. (36) reported that serum anti-PG antibody titers were boosted in patients treated with β-lactam antibiotics but not with vancomycin. Explanations for this phenomenon could be that vancomycin binds and inactivates the sPG released or does not release sPG at all. This is in line with the small TNF release after the treatment of *S. epidermidis* with vancomycin (Fig. 1).

Results obtained with the inhibition ELISA indicated that both sPG and TA, albeit in different concentrations (30-μg/ml and 105-μg/ml PG and TA equivalents, respectively), were present in the supernatants from β-lactam-treated *S. epidermidis* and could account for the TNF secretion (Fig. 2). Although the sPG present in β-lactam supernatants was not completely identical to the purified cell wall PG being used as a coating agent in the ELISA, the immunoglobulin G present in HPS as a detecting agent seems to be directed against common structures, most probably the molecular backbone and the stem peptide, of different bacterial strains (36).

The results of absorption experiments with PMB and vancomycin gels supported the theory that sPG is present in β-lactam supernatants (Table 2). The relatively low absorption efficiency of vancomycin-Sepharose gel for sPG, compared with that of PMB-Sepharose gel for LPS, may be based on differences in affinity. An additional explanation could be that other bacterial products such as TA or lipoteichoic acid contribute to TNF induction. The relatively low TNF-inducing capacity of β-lactam supernatants after the passage of the supernatants over the gels in the absence of serum could be due to nonspecific absorption of sPG.

We have shown before that isolated cell wall PG in the presence of human serum induced TNF by human monocytes independent of the CD14 receptor (18). By using the same monoclonal antibody and assay as before (18), we obtained 8 and 30% inhibition of TNF release induced by β-lactam supernatants in the presence and absence, respectively, of HPS. However, 85% inhibition of TNF release was obtained when LPS (10 ng/ml) in the presence of HPS was used as stimulus (data not shown).

In conclusion, we show that supernatants from β-lactam-incubated *S. epidermidis* induce higher levels of TNF from human monocytes than similar supernatants obtained from *S. epidermidis* incubated with vancomycin or clindamycin or incubated without an antibiotic. Furthermore, we found a correlation between the sPG and TA contents of supernatants and their TNF-inducing capacity, and we propose that sPG and TA are major TNF-inducing components of β-lactam supernatants. Clinical extrapolation of these findings, however, should be made with caution. TNF is essential for an effective host defence against microorganisms in local and sometimes more systemic infections. This fact has been demonstrated by animal models, such as the TNF knockout mouse model of *Listeria* infection (23). Only in special circumstances, e.g., when the cytokine network is no longer properly controlled, can TNF be harmful. Rapid killing of bacteria is probably the most important factor in the clinical outcome of a severe bacterial infection. Penicillin G and oxacillin are well known for their rapid killing, relatively mild side effects, and few interactions with other drugs. Our in vitro data suggest that the choice of antibiotic(s) used in therapy may influence the inflammatory response of the host.

This work was supported by the Bristol Meyer Squibb research foundation.

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Editor: V. A. Fischetti