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Combination of Flucloxacillin and Gentamicin Inhibits Toxic Shock Syndrome Toxin 1 Production by *Staphylococcus aureus* in Both Logarithmic and Stationary Phases of Growth

PETRA VAN LANGEVELDE, JAAP T. VAN DISSEL,* CLAUDIA J. C. MEURS, JASPER RENZ, AND PAUL H. P. GROENEVELD

Department of Infectious Diseases, Leiden University Hospital, Leiden, The Netherlands

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Production of exotoxins by staphylococci and streptococci may lead to the development of toxic shock syndrome (TSS). Because clindamycin inhibits exotoxin production, its use has been advocated for the treatment of TSS. However, the bacteriostatic action of clindamycin might be a disadvantage for the treatment of overwhelming infections. We investigated the effects of flucloxacillin and gentamicin on exotoxin production, because incubation with these antibiotics combines bactericidal action with protein synthesis inhibition. *Staphylococcus aureus* during the logarithmic and stationary phases of growth was incubated with either clindamycin, flucloxacillin, or a combination of flucloxacillin and gentamicin at concentrations of 2 or 10 times the MIC. In logarithmic-phase cultures clindamycin had a static effect on bacterial growth. After incubation with flucloxacillin, either alone or in combination with gentamicin, a rapid and large reduction in the number of viable bacteria was demonstrated. In stationary-phase cultures none of the antibiotics significantly changed the number of viable bacteria. TSS toxin 1 (TSST-1) production during logarithmic-phase growth was inhibited by ≥95% by all antibiotics. In stationary-phase cultures, clindamycin, flucloxacillin, and the combination of flucloxacillin and gentamicin inhibited TSST-1 production by 95, 30, and 75%, respectively, compared with the level of exotoxin production in the controls. The present results indicate that clindamycin inhibits TSST-1 production and exerts bacteriostatic activity in both bacterial growth phases. Because the combination of flucloxacillin and gentamicin combines the inhibition of exotoxin production with high bactericidal activity at least in logarithmic-phase cultures, it should be considered an alternative to clindamycin for the treatment of exotoxin-mediated diseases, especially in patients with overwhelming infections.

Staphylococcal and streptococcal infections can be complicated by the toxic shock syndrome (TSS), which is characterized by high fever, diffuse erythema, arterial hypotension, and multiple organ failure (10). This syndrome is thought to be due to the production of exotoxins that induce extensive T-cell proliferation and cytokine production via a superantigenic mechanism (1, 2). In view of the still high mortality rate among patients with streptococcal TSS, there is a need for new treatment modalities that aim to reduce exotoxin production and inhibit the biological effects of these toxins. In that respect, as far as antimicrobial treatment is concerned, the use of clindamycin instead of β-lactam antibiotics has been advocated as therapy for streptococcal or staphylococcal infections. This is largely based on one in vitro study showing that clindamycin inhibits the production of exotoxin by *Staphylococcus aureus* (7). However, many clinicians are reluctant to use a bacteriostatic antibiotic like clindamycin as empiric therapy in a possibly overwhelming infection. Treatment should focus on both a reduction of exotoxin production and killing of bacteria, especially in streptococcal TSS. In this respect, the effect of a combination of a β-lactam antibiotic with an aminoglycoside, combining bactericidal action with inhibition of protein synthesis, has not been studied.

In the present study a TSS toxin 1 (TSST-1)-producing *S. aureus* strain was used as a model for toxin-mediated illness. The aim of the study was to investigate the in vitro effects of flucloxacillin or gentamicin, or both, compared with those of clindamycin on the number of viable bacteria and exotoxin production. Because the efficacies of antibiotics may depend on the growth phase of the bacteria, the effects of these antibiotics were examined during logarithmic and stationary phases of growth.

MATERIALS AND METHODS

Bacterial strain. A TSST-1-producing *S. aureus* strain designated 89-54, a clinical isolate, was a kind gift of W. J. van Leeuwen (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Bacteria were cultured in brain heart infusion (BHI) broth with aeration at 37°C, conditions optimal for staphylococcal exotoxin production (3, 6). *S. aureus* 89-54 did not produce staphylococcal enterotoxin A, B, or C, as assayed by specific enzyme-linked immunosorbent assays (ELISAs), and the level of TSST-1 production by this strain in BHI medium was similar to the production of exotoxins reported for other *S. aureus* strains (6, 7).

Antibiotics. Flucloxacillin, clindamycin, and gentamicin were purchased from SmithKline Beecham (Herdfordshire, England), Pharmacia-Upjohn (Puurs, Belgium), and Sigma Chemical Co. (St. Louis, Mo.), respectively. The MICs of flucloxacillin and clindamycin were 0.25 mg/liter and the MIC of gentamicin was 1 mg/liter, as determined by standard microdilution techniques.

TSST-1 production during logarithmic and stationary phases of growth in the presence of antibiotics. After overnight culture, *S. aureus* 89-54 was washed twice to remove the TSST-1 in the supernatant. To obtain logarithmic or stationary growth conditions, inocula of 5×10^6 or 1×10^9 bacteria/ml were used, respectively. To reach the logarithmic growth phase or to allow the bacteria to adjust to the medium, the bacteria were cultured for 2 h at 37°C. Flucloxacillin and clindamycin were added to final concentrations of 10 times the MICs, whereas gentamicin was used at 2 and 10 μg/ml (i.e. 2 and 10 times the MIC, respectively), corresponding to the peak and trough levels, respectively, in human serum during antibiotic therapy. At various times after the addition of antibiotics, the numbers of viable bacteria were determined microbiologically and were expressed as the numbers of CFU per milliliter. For quantitation of TSST-1 production, aliquots were centrifuged, filtered, and stored at −20°C until the level of TSST-1 production was measured by ELISA. The level of TSST-1 production in the various incubations was calculated by subtracting the levels at the time of

* Corresponding author. Mailing address: University Hospital Leiden, Department of Infectious Diseases, C5-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31-71-5262613. Fax: 31-71-5266758. E-mail: J.Thompson@thuisnet.leidenuniv.nl.

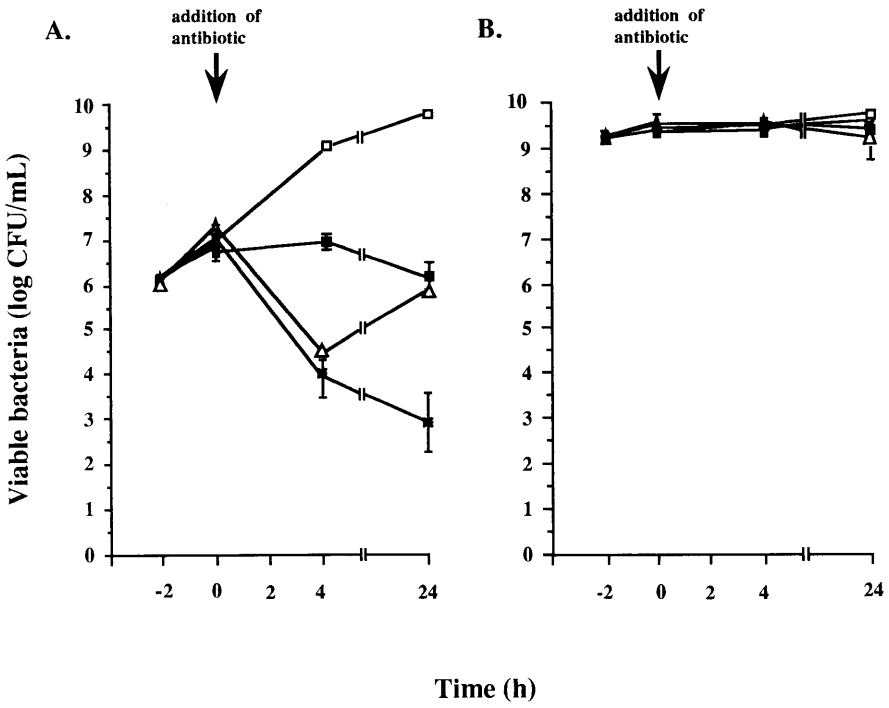

FIG. 1. Number of viable *S. aureus* bacteria in logarithmic-phase (A) and stationary-phase (B) cultures during incubation in the absence (□) or in the presence of different antibiotics. Clindamycin (■) and flucloxacillin (△) were added at concentrations of 2.5 mg/liter (i.e., 10 times the MICs). For the combination of flucloxacillin and gentamicin (✕) concentrations of 2.5 and 2 mg/liters, respectively (i.e., 10 and 2 MIC, respectively), were used. Values are means ± SEMs of three to six experiments.

antibiotic incubation (time zero) from the levels 4 and 24 h thereafter and was expressed as the percentage relative to the level of TSST-1 production by bacteria incubated without antibiotics.

TSST-1 ELISA. The TSST-1 ELISA, as described elsewhere (4), was a kind gift of J. B. Dufrenne (National Institute of Public Health and Environmental Protection). In short, a 96-well Titertek immunoassay plate (ICN Biomedicals B.V., Zoetermeer, The Netherlands) was coated overnight with 2 μg of sheep anti-TSST-1 polyclonal antiserum per ml in buffered saline (pH 8.7). Next, the plates were incubated for 1 h at 37°C with bacterial supernatants, and a standard curve (0 to 50 ng/ml) was made with purified TSST-1 (Toxin Technology, Madison, Wis.). For detection, sheep anti-TSST-1 polyclonal antiserum conjugated with horseradish peroxidase was used. A color reaction was obtained with a substrate of 1 mg of 3,3′,5,5′-tetramethylbenzidine (Sigma) per ml in sodium acetate buffer (pH 6.0) containing 0.006% H_2O_2. The reaction was stopped after 5 min by the addition of 4 N H_2SO_4. The optical density was determined at 450 nm, and the exotoxin concentrations in the supernatants were calculated by using the standard curve for TSST-1. The detection limit of the TSST-1 ELISA was 1.5 ng/ml. Preliminary experiments indicated that the TSST-1 ELISA showed no cross-reactivity with *S. aureus* α-hemolysin and that the antibiotics did not interfere with the color reaction of the ELISA.

Statistical analysis. A Mann-Whitney U test for independent samples was used to analyze the exotoxin production data. Data from three to six experiments were expressed as means ± standard errors of the means, and P values of ≤0.05 were considered significant.

RESULTS

Reduction of the number of viable *S. aureus* bacteria by antibiotics during the logarithmic growth phase. During logarithmic growth of *S. aureus* 89-54 in the absence of antibiotics, the log number of viable bacteria increased from 6.2 ± 0.11 to 7.0 ± 0.11, 8.9 ± 0.08, and 9.7 ± 0.06 at −2, 0, 4, and 24 h of the experiment, respectively (Fig. 1A). For logarithmic-phase cultures of *S. aureus* incubated with clindamycin at a concentration of 10 times the MIC, the drug showed a bacteriostatic effect, whereas flucloxacillin reduced the log bacterial numbers from 7.3 ± 0.07 to 4.5 ± 0.14 in 4 h (Fig. 1A). At 24 h, the log number of bacteria had increased again to 5.8 ± 0.11. This regrowth of *S. aureus* after 24 h of incubation with flucloxacillin at ≤10 times the MIC is probably due to binding of the antibiotic to bacteria or bacterial debris (data not shown). A sustained bactericidal effect was observed with a combination of flucloxacillin and gentamicin (2 times the MIC), which caused a reduction in the number of viable bacteria from 7.0 ± 0.11 to 3.9 ± 0.43 and 2.9 ± 0.65 at 4 and 24 h of incubation, respectively. Preliminary experiments with the combination of flucloxacillin and clindamycin revealed a static effect of the combination on the number of viable bacteria (data not shown).

Reduction of the number of viable *S. aureus* bacteria by antibiotics during the stationary growth phase. The log number of viable *S. aureus* bacteria during the stationary growth phase increased only slightly from 9.2 ± 0.11 to 9.4 ± 0.11, 9.6 ± 0.07, and 9.8 ± 0.04 during the 24 h of the experiment (Fig. 1B). Incubation with either clindamycin, flucloxacillin, or flucloxacillin and gentamicin (2 times the MIC) did not significantly affect the number of viable bacteria under these conditions (Fig. 1B).

Effects of antibiotics on TSST-1 production by *S. aureus* during logarithmic growth. During the logarithmic phase of growth, the amount of TSST-1 produced in control cultures was directly related to the numbers of viable *S. aureus* bacteria (Table 1). Because the bacteria were adjusted to the medium for 2 h before the addition of the antibiotics at 0 h, some TSST-1 was already present at this time point. When logarithmic-phase cultures of *S. aureus* were incubated with antibiotics for 4 h, all antibiotics and antibiotic combinations caused a significant reduction in the level of exotoxin production (Fig. 2A) compared with that by the controls. Incubation with clindamycin and the combination of flucloxacillin and gentamicin completely inhibited TSST-1 production, whereas bacteria incubated with flucloxacillin alone produced a small but just measurable amount of TSST-1 (6.5 ng/ml). At 24 h of incuba-

TABLE 1. Growth of and TSST-1 secretion by *S. aureus* 89-54 during logarithmic and stationary phases[a]

Time (h)	Logarithmic phase		Stationary phase	
	No. of viable bacteria (log_{10} CFU/ml)	TSST-1 production (ng/ml)	No. of viable bacteria (log_{10} CFU/ml)	TSST-1 production (ng/ml)
0	7.01 ± 0.12	2.5 ± 1.8	9.36 ± 0.11	109.4 ± 13.6
4	8.93 ± 0.08	74.7 ± 22.0	9.55 ± 0.07	343.4 ± 62.0
24	9.73 ± 0.06	237.4 ± 44.8	9.78 ± 0.04	446.2 ± 80.9

[a] Data are expressed as means ± SEMs of three to six experiments.

tion, the levels of TSST-1 were below the detection limit (<1.5 ng/ml) for all antibiotics.

Effects of antibiotics on TSST-1 production by *S. aureus* during stationary growth. The level of TSST-1 production during stationary growth in the absence of antibiotics increased rapidly and was already very high after 4 h (Table 1). Culturing for 24 h increased the amount of TSST-1 only slightly over these values. Clindamycin completely inhibited exotoxin production at both time points, whereas flucloxacillin significantly reduced the level of TSST-1 production to 60.3% ± 5.8% at 4 h and 68.6% ± 9.5% at 24 h compared with the level of production by bacteria cultured without antibiotics (Fig. 2B). The combination of flucloxacillin and gentamicin (2 times the MIC) reduced exotoxin levels to 42.7% ± 14.1% and 47.2% ± 10.1% at 4 and 24 h, respectively. This effect depended on the concentration of gentamicin because the combination of flucloxacillin and gentamicin at a concentration of 10 times the MIC reduced the level of TSST-1 production even further to 25.8% ± 5.8% at 4 h and 34.5% ± 6.6% at 24 h. Similar results were obtained with gentamicin only (21.9% ± 10.7% and 31.9% ± 8.7%, respectively).

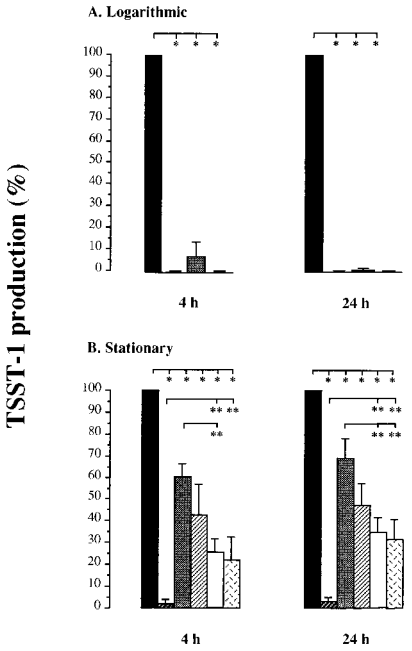

FIG. 2. TSST-1 production by *S. aureus* under logarithmic-phase (A) and stationary-phase (B) culture conditions in BHI medium. Bacteria were cultured in the absence of antibiotics (■) or in the presence of clindamycin (▨), flucloxacillin (▩), a combination of flucloxacillin and gentamicin (▨ and □), or gentamicin (▩) alone. Flucloxacillin and clindamycin were added at concentrations of 2.5 mg/liter (10 times the MIC), and gentamicin was added at concentrations of 2 and 10 mg/liter (2 [▨] and 10 [□ and ▩] times the MIC, respectively). Bacterial culture supernatants were collected by centrifugation and filtration of the cultures, and the level of TSST-1 was measured in a specific TSST-1 ELISA. The level of TSST-1 production was calculated as a percentage relative to the level of TSST-1 production by bacteria incubated without antibiotics. The control level of production of TSST-1 during logarithmic growth was 72.2 and 234.9 ng/ml, and during stationary growth it was 234.0 and 336.8 ng/ml after 4 and 24 h, respectively. Values are means ± SEMs of three to six experiments. *, $P < 0.005$; **, $P < 0.05$.

DISCUSSION

The main findings of our study are that (i) clindamycin completely inhibits TSST-1 production by *S. aureus* during both the logarithmic and stationary phases of growth and (ii) the combination of flucloxacillin and gentamicin also completely inhibits exotoxin production during the logarithmic phase of growth and reduces it by almost 80% during the stationary phase of growth. In logarithmic-phase cultures the reduction of exotoxin production can be caused by either the loss of viability or the inhibition of protein synthesis. Since the reduction of viable bacteria is already near maximal for flucloxacillin only, no distinction can be made between these two possible explanations. In the stationary phase, when the inhibition of bacterial viability is similar for all antibiotics, the effect of the combination of flucloxacillin and gentamicin is probably due to inhibition of protein synthesis by the aminoglycoside. This is confirmed by the finding that gentamicin alone had the same inhibitory effect. Incubation with clindamycin caused an almost total inhibition of exotoxin production, whereas with gentamicin alone, a reduction to about 20% of the level of production in the controls was observed. A possible explanation for this discrepancy could lie in the differences in penetration or uptake of the antibiotics into the bacterial cell. Furthermore, gentamicin and clindamycin bind to different subunits of the bacterial ribosomes (5). This difference in binding specificity might account for the observed discrepancy in exotoxin inhibition. It is unlikely that the observed differences between clindamycin and gentamicin are caused by a change in the level of secretion of intracellularly stored exotoxin, because control experiments showed that less than 5% of the exotoxin is present intracellularly in the presence as well as in the absence of the antibiotics.

Staphylococcal and streptococcal infections can be complicated by the development of TSS. Therapy of staphylococcal TSS should focus on an immediate inhibition of exotoxin production because the clinical severity of this syndrome is mainly toxin mediated and is not the result of an overwhelming infection. In that respect, clindamycin might be the most appropriate choice of antibiotic because it was shown to be a potent inhibitor of exotoxin production in vitro. However, in streptococcal TSS, characterized by overwhelming infection and bacteremia, the treatment should preferably focus on a reduction of both the level of exotoxin production and the number of viable bacteria. In this disease, the combination of a β-lactam and an aminoglycoside might be an alternative to therapy with clindamycin alone because of its strong bactericidal action and its potential to reduce the level of exotoxin production, albeit somewhat less than clindamycin does.

The results indicate that during incubation with antibiotics, bacterial killing of *S. aureus* and exotoxin production depend on the growth phase of the microorganism. These findings extend studies with animals indicating that in a thigh infection model in mice, the bactericidal efficacies of β-lactam antibiotics were significantly reduced after infection with very high inocula of *Clostridium perfringens* (9) and *Streptococcus pyogenes* (8). Under these conditions the mortality rate was lower

in mice treated with clindamycin than in mice treated with benzylpenicillin, presumably due to the inhibition of exotoxin production. On the basis of these findings some clinicians advocate use of the combination of benzylpenicillin and clindamycin for the treatment of streptococcal TSS, whereas an argument against the use of this combination is that the bacteriostatic agent clindamycin inhibits the bactericidal action of benzylpenicillin. Our findings suggest that a regimen with the combination of a β-lactam antibiotic and an aminoglycoside should be investigated in exotoxin-mediated illness as well, because it offers the advantage of potent antimicrobial activity with inhibition of exotoxin synthesis.

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