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Clindamycin Enhances Opsonization of *Staphylococcus aureus*

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Staphylococcus aureus 502A was grown in the presence of one-third of the minimal inhibitory concentration of clindamycin. Phagocytosis of the antibiotic-treated bacteria by human polymorphonuclear leukocytes was significantly enhanced, compared with that of the untreated control ($P < 0.001$). Study of opsonization kinetics by a chemiluminescence assay demonstrated that clindamycin-treated staphylococci were opsonized more rapidly than control bacteria and that the serum concentration required for sufficient opsonization was lower. Complement was consumed much faster, and the opsonic fragment C3b was fixed more rapidly to the bacterial surface when the staphylococci were preincubated with clindamycin. Electron micrographs showed an alteration of the staphylococcal cell wall after clindamycin treatment.

Human polymorphonuclear leukocytes (PMNs), because of their ability to phagocytize and kill microorganisms, serve as the cornerstone of defense against invading bacteria. Several antimicrobial agents have been shown to interfere with the phagocytosis process either directly by influencing the chemotactic, phagocytic, and bactericidal activity of the phagocytic cells or indirectly by inducing changes in the target microorganism (4, 6, 10). Enhanced uptake, killing, or both, of antibiotic-altered bacteria by phagocytes has been reported (1, 2, 5, 8, 11). However, little is known about the mechanisms by which these drugs affect phagocytic functions. Recently it was shown that clindamycin, when present in subinhibitory concentrations, enhances opsonization of *Streptococcus pyogenes* through inhibition of M protein formation (3).

In a previous study, we have investigated the influence of subinhibitory concentrations of several antibiotics on the uptake of *Staphylococcus aureus* (9). It was found that pretreatment with clindamycin and doxycyclin augmented phagocytosis by PMNs. In this investigation, we tried to elucidate the mechanism by which clindamycin affects phagocytosis of *S. aureus* by examining the opsonization kinetics of untreated and clindamycin-pretreated bacteria. This phenomenon was studied by means of leukocyte chemiluminescence (CL) and fixation of the third complement component (C3) to the bacterial surface.

MATERIALS AND METHODS

Bacteria. *S. aureus* 502 A was grown overnight at 37°C in Mueller-Hinton broth (Difco Laboratories) in the presence or absence of one-third of the minimal inhibitory concentration (MIC) of clindamycin (The Upjohn Co.), washed three times in phosphate-buffered saline (PBS), and adjusted photometrically to yield 2×10^8 CFU/ml. For phagocytosis studies, the bacteria were radiolabeled by adding 2 μCi/ml of [*methyl*-^{3}H]thymidine (Amersham Corp.) to the Mueller-Hinton broth.

PMNs. Human PMNs were isolated from heparinized blood by dextran sedimentation and Ficoll-Hypaque gradient, as previously described (9). The purified PMNs were finally suspended in Hanks balanced salt solution with 0.1% gelatin to contain 1×10^7 or 5×10^6 cells per ml.

Phagocytosis assay. The uptake of radiolabeled bacteria by PMNs was measured in an assay that has been described elsewhere (13). The phagocytic mixture contained 5×10^6 PMN, 2×10^7 bacteria, and 5% pooled human serum. After a 15-min incubation at 37°C in a shaking water bath, 100-μl samples were removed to determine leukocyte-associated and total radioactivity by differential centrifugation. In some experiments, the leukocyte pellet was incubated for 30 min in 1 μg lysostaphin (Sigma Chemical Co.) per ml to lyse any noningested leukocyte-adherent staphylococci.

CL. CL response of PMNs during phagocytosis of the bacteria was measured in a liquid scintillation counter (Marc II; Nuclear-Chicago Corp.), operating in the out-of-coincidence mode. The assay mixture contained 1 ml of PMN (5×10^6), 1 ml of preopsonized bacteria (2×10^8), and 0.1 ml of a 0.3 μM luminol

TABLE 1. Mean percentage (±SD) of untreated and clindamycin-treated *S. aureus* taken up by human PMNs after a 15-min incubation of the phagocytic mixture

S. aureus	*n*	Uptake (%)	*P* value
Untreated	8	56.3 ± 9.1	<0.001
Clindamycin treated	8	76.1 ± 5.3	
Untreated + lysostaphin[a]	5	52.0 ± 8.0	<0.01
Clindamycin treated + lysostaphin[a]	5	68.4 ± 5.7	

[a] Leukocyte pellet was incubated in 1 μg of lysostaphin per ml to remove any extracellular leukocyte-adherent staphylococci.

solution. Resting values were determined by substituting Hanks balanced salt solution–0.1% gelatin for bacteria. Initially, the polypropylene vials (Biovials; Beckman Instruments, Inc.) containing PMN and luminol were prewarmed in a shaking water bath at 37°C in the dark. At time zero, opsonized *S. aureus* was added to initiate the reaction. CL was monitored for 0.1 min at 60 s intervals over a period of 20 min. Vials were maintained at 37°C between the counts.

Complement consumption. The tubes containing 0.2 ml of the bacterial suspension (2×10^8 CFU/ml) and 0.8 ml of 10% serum were incubated at 37°C for different time intervals. The total hemolytic complement remaining in the serum was titrated with optimally sensitized sheep erythrocytes by the method of Mayer (7). The consumption of complement in each test sample was expressed as a percentage of the hemolytic complement remaining in a control sample of serum incubated with PBS instead of bacteria.

Measurement of C3 fixation. The amount of C3 fixed to the bacterial surface was measured by using an indirect fluorescent immunoassay. Of the bacterial suspension, 0.2 ml (2×10^8 CFU/ml) was mixed with 0.8 ml of diluted serum and incubated at 37°C in a shaking water bath. At indicated times, the process of C3 fixation was stopped by the addition of 2.5 ml of PBS containing 10 mM EDTA to the vials. Bacteria were washed three times with ice-cold PBS. The pellets were incubated with 500 μl of 1:20 diluted fluorescein isothiocyanate-conjugated antiserum specific for human C3 (Wellcome Research Laboratories) for 15 min at room temperature. After repeating the washing procedure, the pellets were suspended in 2.5 ml of 0.1 N NaOH and left at room temperature for 10 min. After centrifugation, the fluorescence of the supernatant was measured in a spectrofluorometer (Farrand Optical Co.) with excitation and analyzing wavelengths of 485 and 525 nm, respectively. The results are given as percentages ranging from zero (control test performed with PBS) to 100% (maximal emission intensity of the series in a day).

RESULTS

S. aureus grown in the presence of subinhibitory clindamycin concentrations was taken up more readily by PMNs than were control bacteria. After 15 min of incubation, 56% (standard deviation [SD], ±9) of untreated and 76% (SD, ±5) of clindamycin-treated *S. aureus* were phagocytized ($P < 0.001$). The increased uptake was not merely due to enhanced attachment of the bacteria to the PMN, since lysostaphin treatment showed that only about 5% of the bacterial population was attached and not ingested by the PMN, no matter whether the bacteria were drug treated or not (Table 1).

By comparison of the CL responses of the PMNs during phagocytosis of clindamycin-treated and control *S. aureus*, differences in the opsonic requirements of the two bacterial popu-

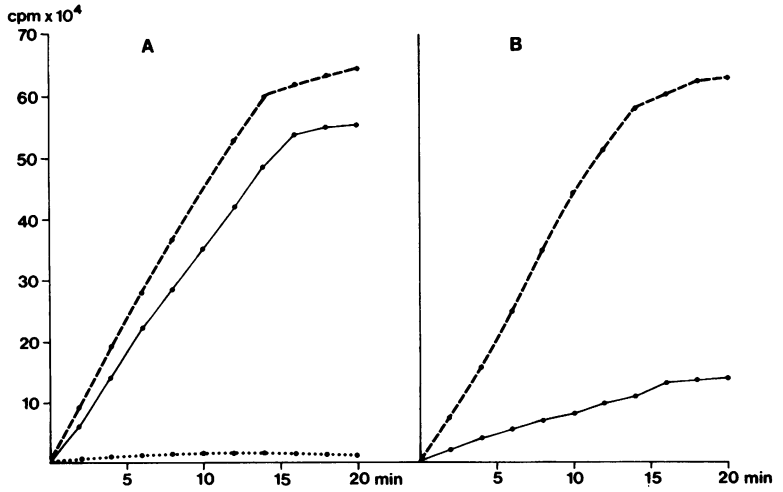

FIG. 1. CL response of PMN induced by untreated (——) and clindamycin-treated (------) *S. aureus*, preopsonized for 5 min in 5% serum (A) and in 1% serum (B). Resting values (. . . .) were obtained by substituting Hanks balanced salt solution–0.1% gelatin for bacteria.

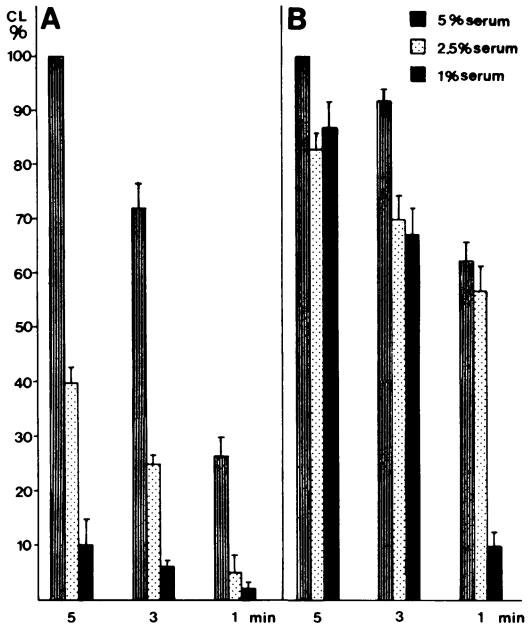

FIG. 2. Peak CL response induced by untreated (A) and clindamycin-treated (B) *S. aureus*, preopsonized for 1, 3, and 5 min in 5%, 2.5%, and 1% serum. The peak values with drug-treated and untreated staphylococci, opsonized for 5 min in 5% serum, were set as 100% in each kinetic experiment. Mean percentage (±SD) of three experiments.

lations were revealed. Clindamycin-treated bacteria, when incubated for 5 min either in 5% serum or in 1% serum, induced the same maximum amount of light emission by the PMNs. In contrast, the CL peak values obtained with untreated *S. aureus* decreased from 550,000 to 130,000 cpm when the serum concentration for opsonization was diminished from 5% to 1% (Fig. 1).

To investigate further the opsonization kinetics, bacteria were preincubated for 1, 3, and 5 min in various serum concentrations. The CL peak values of the PMN induced by drug-treated and untreated staphylococci preopsonized for 5 min in 5% serum were set as 100% in each kinetic experiment.

This manner of presenting the results was chosen because although the CL peak values showed a relatively high day-to-day variation of 20%, the ratio between the absolute kinetic values measured in an experimental series of one day displayed only slight day-to-day variations which never exceeded 5%. As demonstrated in Fig. 2, clindamycin-treated bacteria were already well opsonized after a 1 min exposure to 2.5% serum, inducing about 60% of the CL response. In contrast, untreated control bacteria stimulated only 5% of the CL production.

Since opsonization in heat-inactivated serum resulted in less than 10% phagocytosis of control as well as drug-treated staphylococci, the enhanced opsonization of clindamycin-treated bacteria as measured in the CL assay was assumed to be complement dependent.

Complement consumption of the two bacterial populations was determined by measuring the remaining hemolytic activity in the serum after opsonization of the bacteria for various time intervals. As can be seen in Fig. 3, clindamycin-treated *S. aureus* consumed complement much faster than did control bacteria (for example, 67% versus 16% after 15 min and 97% versus 43% after 30 min of opsonization), the differences being statistically significant ($P < 0.005$).

To examine whether the increased complement consumption by clindamycin-treated *S. aureus* was also reflected by increased binding of the opsonic complement fragment C3b to the staphylococcal cell wall, C3 fixation kinetics were studied by using an indirect immunofluorescent assay. In a previous study, it has been shown that the intensity of the C3-specific fluorescence is directly proportional to the absolute amount of C3 attached to the bacteria (12).

It was assumed that all binding sites for C3 on the bacterial surface were already saturated after 30 min of incubation in 50% serum, as incubation in 100% serum did not further increase the fluorescence intensity. Clindamycin-treated and untreated *S. aureus* fixed the same maximum amount of C3 on the cell wall as

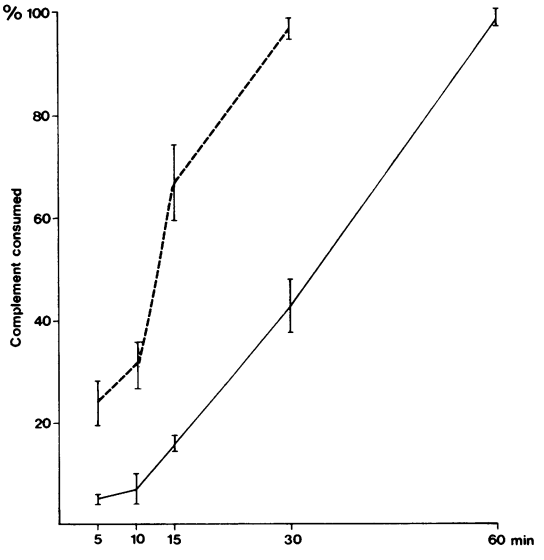

FIG. 3. Mean percentage (±SD) of complement consumed by untreated (——) and clindamycin-treated (------) *S. aureus* during incubation for 5, 10, 15, 30, and 60 min in 10% serum ($n = 3$).

TABLE 2. Percentage of C3 fixed to the surface of untreated and clindamycin-treated *S. aureus* after 1, 3, 5, 15, and 30 min of incubation in various serum concentrations

Incubation time (min)	% Fixation of C3 (mean ± SD)[a] to *S. aureus* in serum concn of:									
	50%		20%		10%		5%		2.5%	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
1	**4.1 ± 1.7**	**27.8 ± 4.3**	**0**	**22.9 ± 3.3**	**0**	13.6 ± 5.5	0	3.2 ± 1.2	0	0
3	**37.8 ± 2.7**	**73.6 ± 2.7**	**10.4 ± 6.8**	**35.4 ± 1.6**	**0**	**27.3 ± 6.2**	0	8.9 ± 3.7	**0**	**8.3 ± 0.4**
5	**45.5 ± 3.2**	**75.5 ± 0.6**	**18.5 ± 4.4**	**45.1 ± 0.1**	**3.0 ± 0.6**	**32.2 ± 1.8**	0.2 ± 0.2	11.5 ± 5.4	**0**	**8.8 ± 1.6**
15	**69.2 ± 3.7**	**86.5 ± 3.7**	**27.1 ± 7.2**	**50.5 ± 2.4**	**17.7 ± 0.2**	**41.0 ± 3.5**	1.5 ± 2.1	15.9 ± 3.5	**0**	**11.1 ± 1.3**
30	**99.2 ± 1.2**	**96.6 ± 4.8**	**54.6 ± 5.3**	**71.0 ± 6.4**	**20.4 ± 0.5**	**48.4 ± 1.5**	**6.7 ± 3.0**	**21.2 ± 1.4**	**0**	**13.7 ± 2.6**

[a] Values are the mean ± SD of two kinetic experiments done on two different days. The maximal emission intensity of the test series of the day was set as 100%.
[b] Boldface numbers indicate that $P < 0.05$ for the treatment pair.

determined in four experiments on four different days, the mean extinction values being, 2,658 (±411) and 2,621 (±309), respectively. Table 2 shows the kinetics of C3 fixation of untreated and clindamycin-treated bacteria in 2.5 to 50% serum. After 1 min of incubation in 50% serum, clindamycin-treated bacteria already bound 28% of C3, compared with only 4% by the control. In 10% serum, drug-treated staphylococci fixed about 15% of C3 after 1 min of incubation, whereas untreated bacteria required 15 min of incubation for binding the same amount of C3.

DISCUSSION

Our study has shown that clindamycin used in a concentration of one-third of its MIC renders *S. aureus* more susceptible to phagocytosis by PMN. The increased uptake of drug-treated bacteria was due to enhanced complement-dependent opsonization. Study of opsonization kinetics in a CL assay demonstrated that clindamycin-treated *S. aureus* was opsonized much faster than untreated bacteria and that the serum concentration required for sufficient opsonization was lower. The enhanced opsonization was reflected by an accelerated complement consumption and C3b binding by the antibiotic-treated staphylococci.

As the maximum amount of C3 fixed by drug-treated and control staphylococci was the same and, in all experiments, an equal number of treated and untreated bacteria was used, we concluded that the maximum C3 binding capacity was the same for each single cell of the two bacterial populations. This indicates that clindamycin does not increase the number of complement-binding sites on the cell wall of *S. aureus*. The increased amount of activated C3 fixed by clindamycin-treated bacteria after short incubation times and in lower serum concentrations, as well as the accelerated complement consumption, suggest an earlier activation of C3 or a higher affinity of activated C3 to the surface of *S. aureus*, or both, after exposure to subinhibitory clindamycin concentrations.

Although clindamycin affects bacterial protein synthesis and is not an antibiotic which acts on the cell wall, it induced alterations of the staphylococcal cell wall structure which appears to be thickened considerably (Fig. 4). It is possible that the morphological changes are responsible for alterations of the complement-activating sites on the bacterial surface.

Gemmell et al. (3) showed that sublethal cocentrations of clindamycin enhance opsonization and phagocytosis of *Streptococcus pyogenes* by affecting formation of M protein on the bacterial surface; the M protein is known to have antiphagocytic properties.

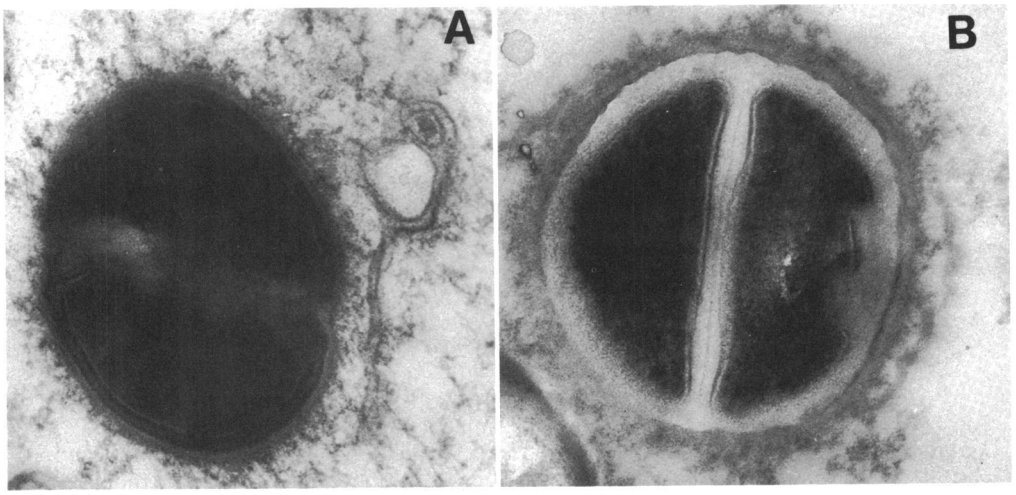

FIG. 4. Electron micrographs of untreated (A) and clindamycin-treated (B) *S. aureus* (magnification, ×40,000).

After exposure to low doses of β-lactam antibiotics, hypersusceptibility of *S. aureus* and group A streptococci to the bactericidal action of leukocytes has been reported (2, 5, 11). The enhanced killing seems not to be a function of increased uptake (9, 11). It was suggested that modification of the bacterial surface structure after antibiotic exposure could be the reason for the increased susceptibility of these cells. Sub-MIC doses of tetracycline were shown to exert a positive effect on the uptake of *S. aureus* (9) and *Listeria monocytogenes* (1). In another study, we have observed an enhanced uptake of staphylococci after exposure to subinhibitory concentrations of erythromycin and chloramphenicol (unpublished data).

It is possible that, in general, antibiotics influencing bacterial surface characteristics, when present in subinhibitory concentrations, facilitate certain steps of the phagocytosis process, the mechanism of action of which remains to be determined for each antibiotic.

As the actual time of contact of bacteria with antibiotic levels greater than the MIC may be relatively short at the site of infection, potentiation of host defense by subinhibitory antibiotic concentrations might also to be expected in vivo.

LITERATURE CITED

1. **Adam, D., W. Schaffert, and W. Marget.** 1974. Enhanced in vitro phagocytosis of *Lysteria monocytogenes* by human monocytes in the presence of ampicillin, tetracycline, and chloramphenicol. Infect. Immun. **9**:811–814.
2. **Friedman, H., and G. H. Warren.** 1974. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after *in vitro* incubation with low doses of nafcillin. Proc. Soc. Exp. Biol. Med. **146**:707–711.
3. **Gemmell, C. G., P. K. Peterson, D. Schmeling, Y. Kim, J. Mathews, L. Wannamaker, and P. G. Quie.** 1981. Potentiation of opsonization and phagocytosis of *Streptococcus pyogenes* following growth in the presence of clindamycin. J. Clin. Invest. **67**:1249–1259.
4. **Hauser, W. E., and J. S. Remington.** 1982. Effect of antibiotics on the immune response. Am. J. Med. **72**:711–716.
5. **Horne, D., and A. Tomasz.** 1981. Hypersusceptibility of penicillin-treated group B streptococci to bactericidal activity of human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **19**:745–753.
6. **Mandell, L. A.** 1982. Effects of antimicrobial and antineoplastic drugs on the phagocytic and microbicidal function of the polymorphonuclear leukocyte. Rev. Infect. Dis. **4**:683–697.
7. **Mayer, M. M.** 1967. Complement and complement fixation. p. 135–153. *In* E. A. Kabat and M. M. Mayer (ed.), Experimental immunochemistry. Charles C. Thomas Publishers, Springfield, Ill.
8. **McDonald, P. J., B. L. Wetherall, and H. Pruul.** 1981. Postantibiotic leukocyte enhancement. Increased susceptibility of bacteria pretreated with antibiotics to activity of leukocytes. Rev. Infect. Dis. **3**:38–44.
9. **Milatovic, D.** 1982. Effect of subinhibitory antibiotic concentrations on the phagocytosis of *Staphylococcus aureus*. Eur. J. Clin. Microbiol. **1**:97–101.
10. **Milatovic, D., and I. Braveny.** 1982. Wechselwirkungen zwischen Antibiotika und Phagozytose. Dtsch. Med. Wochenschr. **107**:1975–1979.
11. **Root, R. K., R. Isturiz, A. Molavi, and J. A. Metcalf.** 1981. Interactions between antibiotics and human neutrophils in the killing of staphylococci. Studies with normal and cytochalasin B-treated cells. J. Clin. Invest. **67**:247–259.
12. **Verbrugh, H. A., W. C. van Dijk, M. E. van Erne, R. Peters, P. K. Peterson, and J. Verhoef.** 1979. Quantitation of the third component of human complement attached to the surface of opsonized bacteria: opsonin-deficient sera and phagocytosis-resistant strains. Infect. Immun. **26**:808–812.
13. **Verhoef, J., P. K. Peterson, and P. G. Quie.** 1977. Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: a quantitative assay using [^{3}H]thymidine labeled bacteria. J. Immunol. Methods **14**:303–311.