SUSCEPTIBILITY OF BACTERIA TO SERUM LYSIS OR PHAGOCYTOSIS FOLLOWING GROWTH IN SUBINHIBITORY LEVELS OF LINCOSAMINIDE OR SPECTINOMYCIN RELATED ANTIBIOTICS

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The effects of subinhibitory concentrations of antibiotics on polymorphonuclear leukocyte (PML) and serum killing of *Staphylococcus aureus* 502A (UC 9116) and *Escherichia coli* UC 9451 were studied. Exposure of these bacteria to subinhibitory levels of certain lincosaminides, spectinomycin, or 6′-*n*-propylspectinomycin altered their susceptibility to these host defense mechanisms, while exposure to gentamicin had no effect. However, each organism responded differently to treatment with the antibiotics. *S. aureus* pretreated during log phase growth with subinhibitory concentrations of clindamycin, lincomycin, or pirlimycin was more susceptible to killing by PMLs than untreated bacteria. No effect on phagocytic killing was found when *S. aureus* was pretreated with spectinomycin, 6′-*n*-propylspectinomycin, or gentamicin. The *S. aureus* remained resistant to serum lysis despite antibiotic treatment. In contrast, spectinomycin and 6′-*n*-propylspectinomycin as well as clindamycin dramatically increased the susceptibility of *E. coli* to serum lysis (>99% destroyed). Moderate killing of *E. coli* by PMLs was also found.

Antibiotics at subinhibitory concentrations (sub-MICs) are capable of altering bacterial morphology, growth rate, toxin production, surface antigens, and adhesion to mucosal surfaces[1~10]. These alterations can affect the invasiveness of bacteria making them more susceptible to host defenses. A cooperative interaction between antibiotics and host defense mechanisms could explain why certain antibiotics exhibit greater efficacy *in vivo* than predicted from their *in vitro* bioactivities. Thus a better understanding of antimicrobial activity *in vivo* might be obtained through the investigation of these interactions. Phagocytosis by polymorphonuclear leukocytes (PMLs) and serum lysis are two primary host responses for eliminating bacteria. In the present study, the effects of sub-MIC levels of lincosaminide and spectinomycin related antibiotics on the susceptibility of bacteria to these two mechanisms were investigated.

Materials and Methods

Antibiotics

Gentamicin sulfate was obtained from Sigma Chemical Company. Lincomycin, clindamycin, and pirlimycin (U-57930E) hydrochlorides were obtained at The Upjohn Company. Pirlimycin is a clindamycin analog in which the (2*S-trans*)-4-*n*-propylhygramide portion of the molecule is replaced by (2*S-cis*)-4-ethylpipecolamide. Spectinomycin dihydrochloride and 6′-*n*-propylspectinomycin sulfate (Trospectomycin, U-63366F) are also Upjohn compounds.

Microorganisms

Staphylococcus aureus 502A (UC 9116) and *Escherichia coli* UC 9451 were used for the experiments. *S. aureus* 502A is a pathogenic strain which survives within leukocytes and is serum resistant. *E. coli* UC 9451 is a clinical isolate obtained from the University of Tennessee Medical School, which is re-

Table 1. The minimum inhibitory concentrations of antibiotics vs. *S. aureus* 502A or *E. coli* UC 9451.

Antibiotic	MIC (μg/ml)	
	S. aureus	*E. coli*
Gentamicin	0.015	0.1
Spectinomycin	40.0	35.0
6′-*n*-Propylspectinomycin	20.0	30.0
Clindamycin	0.15	20.0
Lincomycin	0.80	—
Pirlimycin	0.075	—

sistant to both serum lysis and phagocytosis in our assay. Stock cultures, frozen in 0.5 ml aliquots at −20°C, were used as inoculum for 100 ml of nutrient broth, pH 7.0. Cultures were incubated at 37°C on a rotary shaker at 200 rpm for 4.5~5 hours. Antibiotic was added after 1 hour which corresponded to the beginning of log phase growth. Following incubation, bacteria were washed twice with normal saline by alternate centrifugation at 5,000×*g*. The titer of the suspension was then adjusted to 5×10^7 bacteria per ml using Hanks balanced salts solution lacking Ca, Mg, and phenol red (HBSS) buffered to pH 7.4 with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at 5 mg per ml.

Serum

Fresh autologous, human serum was obtained from clotted blood of healthy volunteers and was diluted to the desired concentration in HBSS/HEPES.

Leukocyte Preparation

PMLs were purified from sodium citrate treated (0.4%) human venous blood by dextran sedimentation. The leukocytes were washed twice using low speed centrifugation in HBSS/HEPES and diluted to 1×10^7 leukocytes per ml in HBSS/HEPES containing 10% autologous serum.

Assay Protocol for *S. aureus*

Phagocytosis or serum lysis of *S. aureus* was determined as follows. Washed bacteria (0.5 ml), treated with antibiotic or untreated, were incubated with either 0.5 ml of serum (10~100%) or 0.5 ml of the leukocytes. The resulting suspension was mixed slowly by rocking for 45 minutes at 37° in a sterile NUNC cryotube (90×12.5 mm). Duplicate tubes were run of each different mixture tested. Colony forming units present in the suspensions were determined before and after incubation.

The samples taken at 0 and 45 minutes were diluted 1: 3 with normal saline and frozen. After thawing, the suspensions were subjected to mild sonic disruption for 5 seconds using a Branson Sonic Oscillator at a power setting of 1. This exposure was sufficient to lyse any remaining intact PMLs and to disrupt clumps of *S. aureus* without killing the bacteria. To quantify the surviving bacteria, each mixture was then diluted in ten-fold increments using normal saline. Two-tenths ml of each dilution was plated onto Trypticase soy agar. Following overnight incubation at 37°C, colony counts were made. Duplicate sets of plates were prepared for each sample and the results averaged.

Assay Protocol for *E. coli*

When *E. coli* was used as the test organism, the assay was performed as described without the freezing and sonication steps. After incubation, the samples were diluted with saline and the viable bacteria were quantified immediately. These *E. coli* do not survive within leukocytes, therefore, lysing the PMLs was not necessary. No change in viable counts occurred after sonic disruption of the leukocytes.

MIC Determination

Since the *S. aureus* and *E. coli* were grown under very specific conditions for these assays, the MIC of each antibiotic vs. these organisms was determined using the same conditions. The MIC values obtained are shown in Table 1. For the assays, the subinhibitory level of antibiotic which was added to the bacterial cultures still allowed a rate of growth similar to untreated cultures. A difference of $\leq$1 log in bacterial titers between control and antibiotic treated cultures was considered acceptable. No gross morphological changes in the antibiotic treated bacteria were obvious microscopically.

Results

Susceptibility of Untreated Bacteria to Serum and PMLs

Serum Susceptibility

Both the *S. aureus* and *E. coli* were tested for their sensitivity to human serum. Bacteria were grown as described and the assays were performed with serum concentrations ranging from 10~100%. No PMLs were added. Both strains of bacteria were totally resistant to even full strength serum. In fact, increases in bacterial titer of approximately 1.5~3-fold were observed within the 45-minute incubation. The bacteria remained resistant to serum through their entire growth cycle.

Leukocyte Susceptibility

The growth phase of bacteria has been reported to affect their susceptibility to phagocytosis[11]. Therefore, bacteria were harvested at different times during log and stationary growth. The bacteria from each time period were mixed with PMLs and assayed as described. As shown in Table 2, *E. coli* was resistant to PML killing during its entire growth cycle. Bacterial cells from log phase cultures grew in the PML suspension during the 45-minute incubation. Although growth did not occur with bacterial cells from 18-hour cultures, these bacteria were still not susceptible to PMLs. PML killing of *S. aureus*, however, was very dependent upon the stage of growth of the bacteria when harvested. The staphylococci became susceptible to PMLs in the late stages of log phase growth with the greatest susceptibility occurring with old stationary phase cultures. *S. aureus* was very resistant to phagocytic killing during early to mid-log phase growth. Total killing of the *S. aureus* was not found since this strain is capable of survival with PMLs. For comparing antibiotic treated and untreated bacteria, cultures from the end of log phase growth (4.5~5 hours) were routinely utilized.

Effect of Pretreatment with Subinhibitory Levels of Antibiotic on Killing of *S. aureus* by Serum or PMLs

Serum Killing

Although *S. aureus* is not typically susceptible to the effects of serum lysis, pretreatment with subinhibitory levels of antibiotic could alter their resistance to this host defense mechanism. *S. aureus* was grown in low concentrations of three lincosaminides, spectinomycin, 6′-*n*-propylspectinomycin, and gentamicin. After washing to remove the antibiotics, the bacteria were incubated with full strength serum as described. As shown in Table 3, no change in serum sensitivity occurred after growth in the presence of these antibiotics. Untreated as well as antibiotic treated bacteria were totally resistant to serum.

Leukocyte Killing

Killing of *S. aureus* by PMLs after growth in subinhibitory levels of three lincosaminide antibiotics was enhanced compared to untreated bacteria. Increases in phagocytic killing of 10~60% were observed depending on donor sera and PMLs. Spectinomycin, 6′-*n*-propylspectinomycin, and gentamicin were ineffective in enhancing

Table 2. Effect of growth phase upon PML killing of *S. aureus* 502A or *E. coli* UC 9451[a].

Time harvested (hours)	Survival (%)[b]	
	S. aureus	*E. coli*
2	169	390
3.5	350	300
5	48	200
18	28	100

[a] PMLs (10^7/ml) in 10% serum were incubated with *S. aureus* ($5\sim8\times10^7$/ml) for 45 minutes.

[b] Results are expressed as percentages of the viable number of bacteria at zero time surviving after 45 minutes and are the mean of 3 or more experiments.

Table 3. Effect of serum on *S. aureus* 502A treated with subinhibitory concentrations of antibiotics[a].

Antibiotic	Concentration (μg/ml)	Sub-MIC level	Viable bacteria (%)[b]
None	—	—	225
Clindamycin	0.05	1/3	194
Lincomycin	0.27	1/3	200
Pirlimycin	0.025	1/3	198
Spectinomycin	8.0	1/5	192
6′-*n*-Propylspectinomycin	4.0	1/5	189
Gentamicin	0.005	1/3	210

[a] Bacteria ($5\sim8\times10^7$/ml) were incubated with full strength serum for 45 minutes.
[b] Results are expressed as percentages of the viable number of bacteria at zero time surviving after 45 minutes and are the mean of 3 or more experiments.

Table 4. Enhancement of PML killing of *S. aureus* 502A treated with subinhibitory concentrations of antibiotics[a].

Antibiotics	Concentration (μg/ml)	Sub-MIC level	Viable bacteria (%)[b] (mean±SD)
None	—	—	75.0±13.9
Clindamycin	0.05	1/3	40.9±11.6
Lincomycin	0.27	1/3	38.5±6.4
Pirlimycin	0.025	1/3	43.5±9.2
Spectinomycin	8.0	1/5	76.8±10.3
6′-*n*-Propylspectinomycin	4.0	1/5	77.7±11.5
Gentamicin	0.005	1/3	75.2±14.2

[a] PMLs (10^7/ml) in 10% serum were incubated with *S. aureus* ($5\sim8\times10^7$/ml) for 45 minutes.
[b] Results are expressed as percentages of the viable number of bacteria at zero time surviving after 45 minutes and are the mean of 3 or more experiments.

phagocytic destruction. PML killing of *S. aureus* grown with clindamycin, lincomycin, or pirlimycin averaged between 56~61%. Only 22~25% of *S. aureus* grown with the spectinomycin antibiotics or gentamicin were destroyed by phagocytosis which was identical to that observed for untreated bacteria. These results are summarized in Table 4.

Effect of Pretreatment with Subinhibitory Levels of Antibiotic on Killing of *E. coli* by Serum or PMLs

Serum Killing

This particular pathogenic strain of *E. coli* (UC 9451) is resistant to lysis by serum components. Gram-negative bacteria can be very susceptible to serum lysis, however. Therefore, antibiotics might be capable of altering the susceptibility of a serum resistant strain such as the one utilized. The *E. coli* was grown in subinhibitory levels of clindamycin, the spectinomycins, and gentamicin. The serum killing assay was performed as described with varying concentrations of serum diluted in HBSS/HEPES. Significant differences in serum susceptibility were found following antibiotic treatment as seen in Table 5. Very little effect was detectable even in full strength serum when *E. coli* was grown in sub-MIC levels of gentamicin. However, upon exposure to sub-MIC levels of clindamycin, spectinomycin, or 6′-*n*-propylspectinomycin, >99% of the *E. coli* was destroyed by serum lysis. This effect was concentration dependent with over 50% killing occurring with serum concentrations ≦10%.

Leukocyte Killing

PML preparations were routinely made with 10% serum as the source of opsonins. Since serum

Table 5. Enhancement of serum killing of *E. coli* UC 9451 treated with subinhibitory concentrations of antibiotics[a].

Antibiotic	Concentration (μg/ml)	Sub-MIC level	Viable bacteria (%)[b]		
			Serum (%)		
			10	20	100
None	—	—	196	182	190
Clindamycin	7.0	1/3	41	<1	<1
Spectinomycin	7.0	1/5	46	<1	<1
6′-*n*-Propylspectinomycin	6.0	1/5	50	<1	<1
Gentamicin	0.01	1/10	240	190	83

[a] Bacteria ($5\sim8\times10^7$/ml) were incubated for 45 minutes with serum diluted to the desired concentration with HBSS/HEPES.

[b] Results are expressed as percentages of the viable number of bacteria at zero time surviving after 45 minutes and are the mean of 3 or more experiments.

Table 6. Enhancement of PML killing of *E. coli* UC 9451 treated with subinhibitory concentrations of antibiotics[a].

Antibiotic	Concentration (μg/ml)	Sub-MIC level	Killing by PMLs (%)[b] (mean±SD)
None	—	—	0
Clindamycin	7.0	1/3	10±1.7
Spectinomycin	7.0	1/5	17±5.0
6′-*n*-Propylspectinomycin	6.0	1/5	13±2.8
Gentamicin	0.01	1/10	0

[a] PMLs (10^7/ml) in 10% serum were incubated with *E. coli* ($5\sim8\times10^7$/ml) for 45 minutes.

[b] Percent killing by PMLs was determined to be the difference in viable counts between control mixtures containing 10% serum and bacteria and those containing serum, bacteria, and PMLs (see text). A minimum of 3 experiments were run for each antibiotic.

killing of *E. coli* was still found at this level, controls containing only serum and bacteria were run with each antibiotic tested. Killing by PMLs was considered to be the difference in viable counts between mixtures containing only serum and those containing serum plus PMLs. As shown in Table 6, phagocytosis was observed after treatment with clindamycin, spectinomycin, and 6′-*n*-propylspectinomycin. Although the levels of killing were only moderate, they were consistently observed in each test. No destruction by PMLs was detected with untreated bacteria or those treated with gentamicin.

Discussion

These studies, along with others[12~16], indicate that at low levels antibiotics can produce changes in bacteria which increase their susceptibility to host defense mechanisms *in vitro*. The effects observed were brought about by alteration of the bacteria, since all antibiotics were removed before exposure of the bacteria to serum or PMLs. These changes did not affect the viability of the bacteria, but did increase their susceptibility to host defenses. The mechanisms involved appear to vary depending on the specific microorganisms and antibiotic involved. Clindamycin, and the other lincosaminides, were the only antibiotics tested which were effective in increasing the susceptibility of both *S. aureus* 502A and *E. coli* UC 9451 to phagocytic destruction by PMLs. Although not dramatic, the increases in killing from 10~60% were in the range reported in other studies[17~20]. Spectinomycin and its analog, 6′-*n*-propylspectinomycin, were not effective against *S. aureus*, but did have limited effectiveness against *E. coli*. An increase in PML killing of approximately 15% was found. None of the antibiotics tested

were capable of altering the serum resistance of the *S. aureus*. However, significant changes in serum sensitivity were found after exposure of the *E. coli* to clindamycin, spectinomycin, and 6′-*n*-propyl-spectinomycin. Untreated *E. coli* were totally resistant to serum, whereas, 99% of those grown in the presence of subinhibitory levels of these antibiotics were killed in 20% serum. Gentamicin had no effect against either the *S. aureus* or the *E. coli* and served as a negative control. These results are consistent with other reports involving gentamicin[11,21,22].

Augmentation of host defense mechanisms by antibiotics could explain discrepancies between *in vivo* efficacy and *in vitro* bioactivities. It could also explain why therapeutic effects can be obtained even when the serum levels of some antibiotics fall below their MIC. Thus, assays which measure modulation of host defense mechanisms, such as the one described, may prove useful as early indicators of *in vivo* efficacy. Although the clinical significance of these observations remains to be established, investigation of synergistic effects between antibiotics and host defense mechanisms should provide a better understanding of the potential antimicrobial action of antibiotics *in vivo*.

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