AUGMENTATION OF SERUM BACTERICIDAL ACTIVITY BY PALDIMYCIN

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At concentrations below the MIC, paldimycin induced changes in *Staphylococcus aureus* 502A (UC 9116, ATCC 28417) which increased its sensitivity to serum. The enhanced sensitivity to serum was concentration dependent with the maximal sensitivity found when bacteria were grown in approximately 1/10 MIC of paldimycin. Within an 1-hour incubation, *S. aureus* 502A typically grew 1.5~2-fold in serum. Following exposure to paldimycin, however, approximately 30~50% of the bacteria were killed in serum. The paldimycin treated bacteria were not more susceptible to phagocytosis and killing by polymorphonuclear leukocytes. At the concentrations utilized, the Staphylococci were enlarged and had thickened cell walls. The organisms were still viable and replicating, but irregularities in cell division were observed in transmission electron micrographs.

Sublethal concentrations of antibiotics can affect bacterial morphology and surface determinants, toxin production, adhesion, and their rate of growth. These antibiotic-induced changes in bacteria can enhance their susceptibility to various host defense mechanisms[1~10]. Direct correlations between the ability of antibiotics to augment host defenses and clinical efficacy have not definitively been established. Nevertheless, a better understanding of an antibiotic effect *in vivo* might be obtained through the investigation of antibiotic/host defense interactions. In the present study, the effects of subinhibitory levels of paldimycin on the susceptibility of *Staphylococcus aureus* 502A (UC 9116, ATCC 28417) to killing by serum lysis or polymorphonuclear leukocytes (PMLs) were studied. Numerous antibiotics have been shown to enhance either one or both of these host defense mechanisms, including the lincosaminides, the spectinomycins[11~14], certain β-lactams[15~20], chloramphenicol[20,21], tetracycline[20], and vancomycin[17]. Gentamicin was found to have no effect[12,22,23]. Paldimycin has a novel structure, however, and so its ability to enhance these mechanisms was of interest. Paldimycin is structurally related to the paulomycins which were originally isolated from fermentations of *Streptomyces paulus*[24,25]. It has *in vitro* and *in vivo* activity against a variety of pathogenic organisms[26,27].

Materials and Methods

Chemicals

Paldimycin (U-70138F) and clindamycin (U-21251F) were obtained at The Upjohn Company. HBSS/HEPES buffer was prepared by the addition of 10% Hanks balanced salt solution (10× concentrated without calcium, magnesium, phenol red, and sodium bicarbonate) to 0.02 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4.

Microorganism and Growth Conditions

S. aureus 502A (UC 9116, ATCC 28417) was used for all experiments. This organism exhibits high level resistance to serum lysis and PML killing *in vitro*[11,28]. Stock cultures, frozen in 0.5 ml

aliquots at −20°C, were used as inoculum for 100 ml 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 and resuspended in HBSS/HEPES to $7\sim10\times10^7$ cfu/ml.

Serum

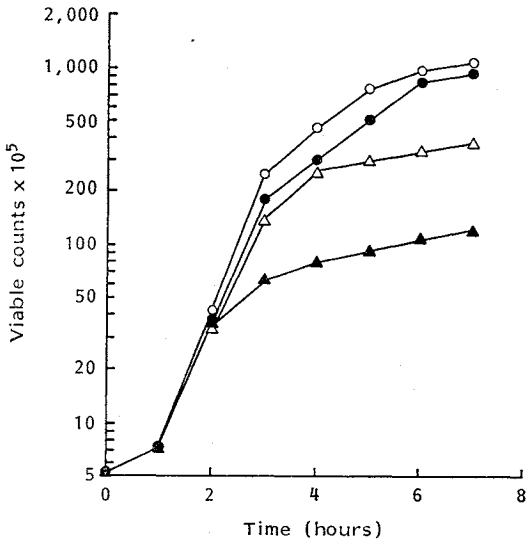
Normal human serum was diluted to the desired concentration in HBSS/HEPES.

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.

Fig. 1. Growth of *Staphylococcus aureus* 502A in varying concentrations of paldimycin.

Paldimycin (μg/ml): ○ 0, ● 0.1, △ 0.2, ▲ 0.3.



Assay Protocol

Phagocytic killing 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 serum (10~100%) or 0.5 ml of the leukocytes in 10% serum (see above). The resulting suspension was mixed slowly by rocking for 45 minutes at 37°C in a sterile Nunc cryotube (90×125 mm). Duplicate tubes were prepared for each different mixture tested. The cfu present in the suspensions were determined before and after incubation. Before quantifying the surviving bacteria, the suspensions containing PMLs were subjected to mild sonic disruption for 5 seconds using a Bronson Sonic Oscillator at a power setting of 1. This exposure was sufficient to lyse intact PMLs and to disrupt clumps of *S. aureus* without killing the bacteria. This procedure was not necessary in assay mixtures containing only serum and bacteria.

MIC Determination

The MIC of paldimycin was determined using the growth conditions described, except the cultures were grown for 18 hours. The MIC value obtained was 3.0 μg/ml. As shown in Fig. 1, the organism can grow in the presence of paldimycin at levels below the MIC, but at a slower rate than control cultures.

Transmission Electron Microscopy

For electron microscopic examination, samples were pelleted by centrifugation then fixed with 3% glutaraldehyde in 0.1 M cacodylate (pH 7.2), and allowed to fix for 1 hour at room temperature. Following 3 rinses in buffer (0.1 M cacodylate, pH 7.2), cells were post-fixed in 1% osmium tetroxide in buffer for 1 hour. Cells were then rinsed 3 times with distilled water, and stained for 30 minutes in 1% aqueous uranyl acetate. Following 2 water rinses, cells were dehydrated with graded ethanols through 100%, cleared in propylene oxide, and embedded in Polybed 812 (Polysciences, Inc.). Thin sections were cut with diamond knives on an MT-5000 ultramicrotome (Dupont-Sorvall), stained with Reynolds lead citrate[29], viewed and photographed on a Jeol 1200EX scanning-transmission electron microscope.

Results

Effect of Paldimycin on the Susceptibility of *S. aureus* to Serum

S. aureus 502A is resistant to the bactericidal action of serum. When washed, untreated Staphy-

Table 1. Susceptibility of *Staphylococcus aureus* 502A to serum killing following growth in subinhibitory levels of paldimycin[a].

Expt No.	Viable bacteria (%)[b]			
	Paldimycin (μg/ml)			
	0	0.1	0.2	0.3
1	147	104	62	32
2	176	136	114	83
3	150	122	100	75
4	215	134	95	65
5	104	85	70	53
6	129	90	75	58
7	242	112	87	67
8	171	94	65	40
Mean±SD	166.8±44.9	109.6±19.7	83.5±18.5	59.1±17.2

[a] Bacteria ($5\sim8\times10^7$/ml) were incubated with serum (50%) for 45 minutes.
[b] Results are expressed as percentages of the viable number of bacteria at 0 time surviving after 45 minutes incubation with serum.

Table 2. Effects of paldimycin on killing of *Staphylococcus aureus* 502A by polymorphonuclear leukocytes (PMLs)[a].

Growth of bacteria (hours)	Viable bacteria (%±SD)[b]			
	Paldimycin (μg/ml)			
	0	0.1	0.2	0.3
5	131±5.6	120±12.4	150±14.8	165±20.6
4	53.3±12.4	53.3±10.8	60.3±12.8	67.1±17.1

[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 0 time surviving after 45 minutes incubation and are the mean of 3 or more experiments.

lococci were incubated with serum, an increase in cfu of approximately 2-fold occurred within 1 hour. Bacteria grown in subinhibitory levels of paldimycin, however, became susceptible to serum lysis as shown in Table 1. The bactericidal activity of the serum varied in each experiment, but killing was consistently found with *S. aureus* exposed to 1/10 MIC (0.3 μg/ml) of antibiotic. When lower concentrations of paldimycin were utilized, growth in serum was always inhibited, but killing was not observed in every experiment.

Effects of Paldimycin on the Killing of *S. aureus* by PMLs

S. aureus 502A is phagocytosed by PMLs, but a small percentage do survive within the phagolysosome and multiply[27]. The *in vitro* susceptibility of the organism to PML killing is dependent upon the growth phase[11]. Table 2 shows that 5-hour cultures are resistant to PML killing, whereas an average of 53% of the bacteria in 4-hour cultures are killed within a 45-minute incubation with PMLs. Pretreatment with paldimycin did not alter the susceptibility of either a 4- or 5-hour culture. No enhancement of killing was detectable.

Comparison of Clindamycin and Paldimycin in Enhancing Host Defense Mechanisms

Clindamycin has been widely tested for its ability to augment host defense mechanisms[8,11~14].

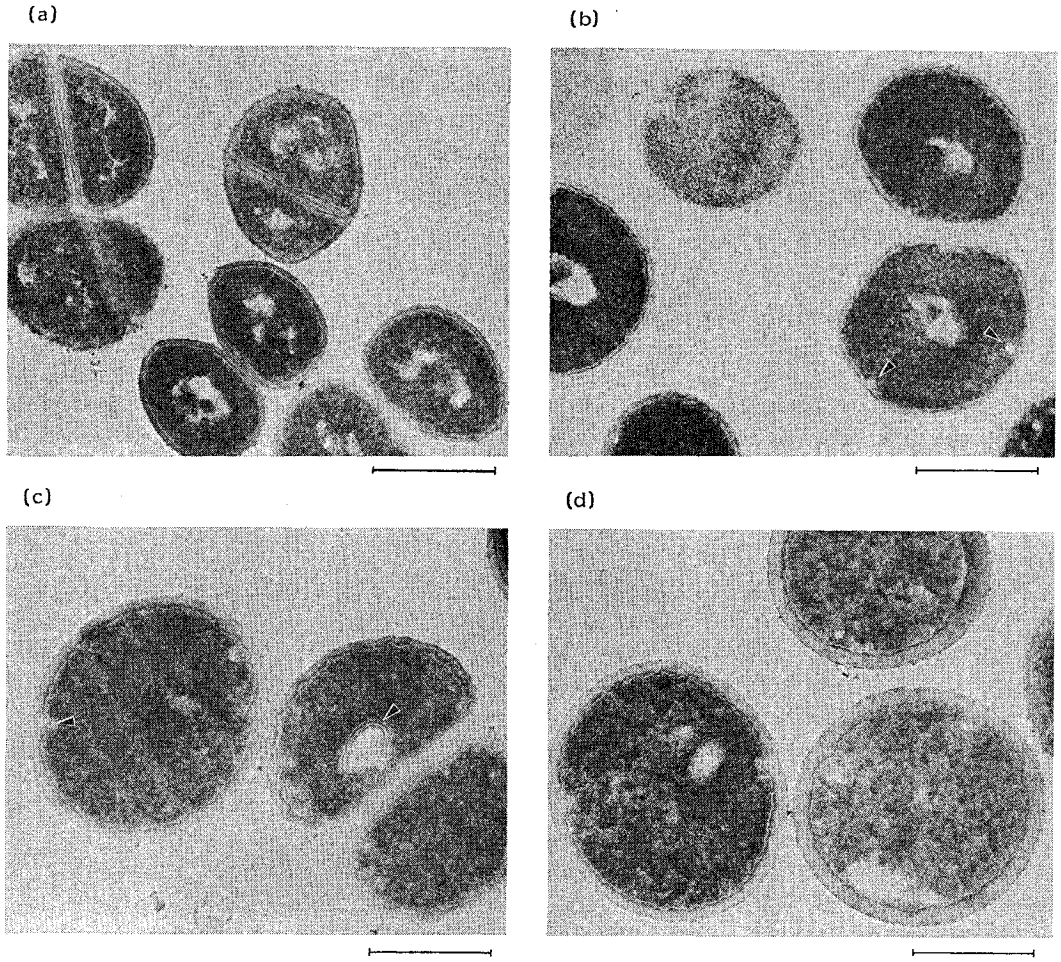
Table 3. Comparison of clindamycin and paldimycin in altering serum or polymorphonuclear leukocyte (PML) killing of *Staphylococcus aureus* 502A[a].

Antibiotic	Concentration (μg/ml)	Sub-MIC level	Viable bacteria (%±SD)[b]	
			Serum	PMLs
None	—	—	143±20.4	75±13.9
Clindamycin	0.075	1/3	134±25.1	38.5±6.4
Paldimycin	0.300	1/10	53.1±10.9	67±9.9

[a] Bacteria ($5\sim8\times10^7$/ml) were incubated with either 50% serum or PMLs (10^7/ml) in 10% serum for 45 minutes.

[b] Results are expressed as percentages of the viable number of bacteria at 0 time surviving after 45 minutes incubation and are the mean of 5 or more experiments.

Fig. 2. Morphology of *Staphylococcus aureus* 502A grown in varying concentrations of paldimycin observed using transmission electron microscopy.



Arrows indicate examples of irregular cell structure and division.

(a) A 5-hour culture grown in the absence of paldimycin. (b) A 5-hour culture grown in 0.1 μg/ml paldimycin. (c) A 5-hour culture grown in 0.2 μg/ml paldimycin. (d) A 5-hour culture grown in 0.3 μg/ml paldimycin.

Bar represents 0.5 μm.

Enhanced phagocytic killing of bacteria following growth in subinhibitory levels of clindamycin has been repeatedly shown. However, clindamycin has been reported to have no effect on the serum sensitivity of *S. aureus*[11]. Therefore, as a control experiment, clindamycin and paldimycin were tested simultaneously in our assay (Table 3). As expected, enhanced PML killing of *S. aureus* grown with 1/3 MIC of clindamycin was found. No enhancement was observed when *S. aureus* was grown in 1/10 MIC of paldimycin. However, the susceptibility of the Staphylococci to serum increased following exposure to paldimycin, but not to clindamycin. Clindamycin-treated bacteria remained resistant to serum.

Electron Micrographs of *S. aureus*

Morphological alterations in *S. aureus* following exposure to varying concentrations of paldimycin were observed by transmission electron microscopy (Fig. 2). Following exposure to paldimycin in nutrient broth, bacteria were found to increase in diameter in a dose-dependent manner when compared to untreated control cultures. Additionally, considerable thickening of the cell wall was observed. At the highest concentration tested (Fig. 2d), cells were approximately 2-fold larger than controls (0.54 μm in controls to 0.96 μm in treated), and cell wall-thickness was approximately 4-fold greater (1.5 nm in controls to 6.2 nm in treated). Few indications of cell division were observed in the highest level of paldimycin. At lower concentrations, abnormalities in cell division were apparent (Figs. 2b and 2c). Peripheral mesosomes were frequently observed also.

Discussion

Although the main therapeutic effect of an antibiotic is to kill bacteria or inhibit their growth, the results of this study and those of other investigations[8~23] suggest that even at subinhibitory levels, certain antibiotics can alter bacteria making them more susceptible to host defenses. Such low levels of antibiotic may be found in serum after peak titers have fallen or at tissue sites of infection. Paldimycin at sub-MIC levels augmented serum killing of *S. aureus* 502A. An average of 41% of the bacteria were killed following exposure to only 1/10 MIC of the antibiotic. At lower levels, their growth in serum was inhibited or greatly reduced compared to untreated bacteria. This rate of serum killing is similar to that found when *S. aureus* (ATCC 25923) was exposed to subinhibitory levels of cefamandole[16].

Paldimycin has been demonstrated to inhibit protein synthesis[30]. The cell wall thickening and the irregularities in cell division observed in the Staphylococci exposed to paldimycin have been observed using other antibiotics with this mode of action, such as the lincosaminides and certain macrolides, and is also characteristic of starvation for a non-peptidoglycan essential amino acid[16,31,32]. Antibiotic induced morphological changes in bacteria, such as these, which alter surface characteristics have been associated with increased susceptibility to various host defense factors, including serum[13,16,33]. Although Gram-positive bacteria are not generally sensitive to the bactericidal activity of serum, the ultrastructural changes induced by treatment with paldimycin could alter the antigenicity of the *S. aureus* 502A thus making them susceptible to serum.

Augmentation of host defense mechanisms by antibiotics, such as observed with paldimycin, could explain why certain antibiotics exhibit greater efficacy *in vivo* than predicted from *in vitro* bioactivities. 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 antimicrobial activity of an antibiotic *in vivo*.

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