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Mechanism of Penicillin-Erythromycin Synergy on Antibiotic-Resistant *Staphylococcus aureus*

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Clinically isolated strains of *Staphylococcus aureus* that are inducibly resistant to both erythromycin and penicillin were susceptible to a combination of the two antibiotics. The synergistic effect of the combination results from an inhibition of penicillinase induction by erythromycin, sparing penicillin and allowing this drug to inhibit growth. When resistance to erythromycin is constitutive rather than inducible, the combination is no longer synergistic.

Penicillin combined with a second antibiotic has been reported to be effective against penicillinase-producing bacteria (9-11, 14, 15). The second drug in these cases is often an inhibitor of protein synthesis, capable of inhibiting the biosynthesis of a β-lactamase, thereby "protecting" the penicillin molecule from destruction. The combinations are effective against many penicillin-resistant strains, yet most reports describe only strains that retain susceptibility to the second antibiotic.

Staphylococcus aureus 1206 is a natural isolate that is inducibly resistant to erythromycin and other macrolide, lincosamide, and streptogramin B-type antibiotics (1, 17). The mechanism for this resistance has been explained as an erythromycin-induced methylation of 23S ribosomal ribonucleic acid that results in macrolide, lincosamide, and streptogramin B-type antibiotic-resistant ribosomes (6, 16). This strain also produces an inducible β-lactamase (1) and is resistant to benzylpenicillin and other penicillinase-sensitive penicillins. In our studies with this strain, we discovered that, although resistant to relatively high concentrations of both erythromycin and benzylpenicillin when tested individually, *S. aureus* 1206 was unusually susceptible to a combination of the two antibiotics. This synergism appears similar to that described earlier (4, 5, 12, 14, 15) for resistant strains of *S. aureus*. We report here that the effectiveness of the benzylpenicillin-erythromycin combination against *S. aureus* can be explained as one drug (erythromycin) interfering with the development of resistance to the other (benzylpenicillin) and that mutation from inducible to constitutive erythromycin resistance abolishes the synergy. (A preliminary report of this work was presented at the 75th Annual Meeting of the American Society for Microbiology, New York, April 27-May 2, 1975.)

MATERIALS AND METHODS

Bacterial strains. *S. aureus* 1206 and a constitutive erythromycin-resistant mutant derived from it (1206 C4) have been described previously (1). All other strains of *S. aureus* are natural isolates of clinical origin demonstrating resistance to both benzylpenicillin and erythromycin. These strains, originally obtained through the Lilly Laboratory for Clinical Research, were kindly provided by C. Godzeski and D. Preston. Inducible β-lactamase was determined by the method described by Duma and Kunz (3), using 2(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid as an inducer. Erythromycin resistance was characterized as inducible or constitutive by the disk method as described by Weisblum and Demohn (17).

Growth media. A synthetic growth medium (1) was used in most of the experiments reported here. A tryptone-glucose medium (18) was used where indicated.

Antibiotic susceptibility. Susceptibility to benzylpenicillin, erythromycin A, and combinations of these two antibiotics was determined by serially diluting antibiotics (twofold dilutions) in either synthetic or tryptone-glucose broth. The inoculum was 0.05 ml of an overnight Trypticase soy broth culture diluted so that the final concentration in each dilution was approximately 10^5 bacteria per ml. The minimal inhibitory concentration was determined as the lowest concentration preventing visible growth after 18 to 24 h of incubation at 37°C.

RESULTS

The minimal inhibitory concentrations for benzylpenicillin and erythromycin when tested separately against *S. aureus* 1206 were equal to or greater than 256 μg/ml. However, 4 μg each of benzylpenicillin and erythromycin per ml, in combination, completely inhibited growth. Identical minimal inhibitory concentrations were obtained by using a synthetic or a tryptone-glucose broth.

The inducible nature of benzylpenicillin and erythromycin resistances is shown in Fig. 1. Ben-

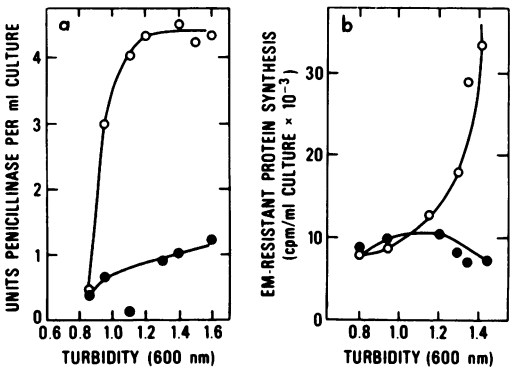

FIG. 1. *Induction of penicillin and erythromycin resistances in S. aureus 1206. (a) Induction of penicillinase. Duplicate cultures were grown in synthetic broth at 37°C to mid-exponential phase. At this time, benzylpenicillin was added to one flask at 1 μg/ml to induce penicillinase. The second flask received no inducer. Samples (5 ml) were removed as indicated for turbidity and penicillinase measurements. Induction was stopped by adding chloramphenicol (200 μg/ml) to the sample, and penicillinase was measured iodometrically by the method of Perret (13), using benzylpenicillin as a substrate. One unit of enzyme activity is defined as that amount of enzyme needed to hydrolyze 1.0 μmol of benzylpenicillin in 1 h at 30°C. (b), Induction of erythromycin-resistant protein synthesis. Duplicate cultures were grown and induced as described for penicillinase induction except that the inducer added to one flask was erythromycin at 0.025 μg/ml. Samples (5 ml) were removed as indicated for measuring turbidity and [^{14}C]leucine incorporation. Rates of [^{14}C]leucine incorporation in the presence of 100 μg of erythromycin per ml were measured on washed cells exactly as described previously (1). Symbols: ○, inducer added; ●, no inducer added.*

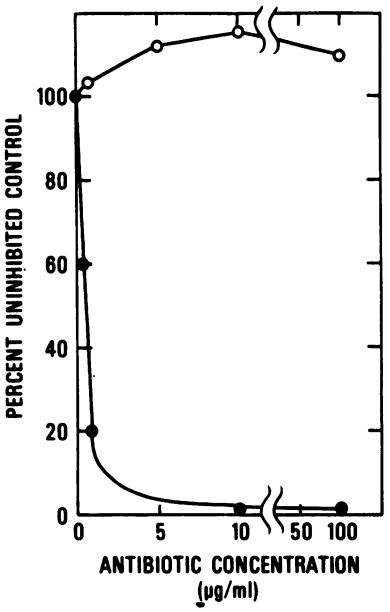

FIG. 2. *Effect of erythromycin on the induction of penicillinase and effect of penicillin on the induction of erythromycin resistance. Cultures were grown and induced essentially as described in the legend to Fig. 1. Penicillinase was induced by adding benzylpenicillin (1 μg/ml) to a series of flasks containing exponentially growing cells preincubated for 5 min in the presence or absence of erythromycin (0 to 100 μg/ml). Incubation was continued for 60 min, and penicillinase was measured in each flask as described in the legend to Fig. 1. Likewise, erythromycin-resistant protein synthesis was induced by adding erythromycin (0.025 μg/ml) to a series of flasks containing exponentially growing cells preincubated for 5 min in the presence or absence of benzylpenicillin (0 to 100 μg/ml). Incubation was continued for 120 min, and erythromycin-resistant [^{14}C]leucine incorporation was measured in each flask as described in the legend to Fig. 1. The data are presented as percentages of control values measured on cells induced in the absence of added inhibitors. Symbols: ●, effect of erythromycin on induction of penicillinase; ○, effect of benzylpenicillin on induction of erythromycin-resistant [^{14}C]leucine incorporation.*

zylpenicillin at 1 μg/ml induced an approximate 4.5-fold increase in the biosynthesis of penicillinase in this strain. Erythromycin, at 0.025 μg/ml, induced erythromycin resistance as measured by the development of erythromycin-resistant [^{14}C]leucine incorporation into protein. In the absence of the appropriate inducers, levels of penicillinase and erythromycin-resistant incorporation showed little change.

The fact that resistance to both antibiotics is inducible raised the possibility that the effectiveness of the combination might be due to one drug interfering with induction of resistance to the other. The effect of penicillin on induction of erythromycin-resistant protein synthesis and the effect of erythromycin on induction of penicillinase were determined, and the data are shown in Fig. 2. Erythromycin had a pronounced inhibitory effect on induction of penicillinase, but benzylpenicillin, even at 100 μg/ml, failed to suppress development of erythromycin resistance. The inhibition of penicillinase by erythromycin concentrations as low as 0.1 μg/ml was due to an effect on enzyme synthesis because the enzymatic activity of penicillinase appears unaffected by much higher concentrations of erythromycin (1). It is obvious that erythromycin is an effective inhibitor of protein synthesis under these conditions due to the fact that resistance to this antibiotic develops more slowly than resistance to benzylpenicillin (Fig. 1).

The inhibitory effects of erythromycin on the induction of penicillinase are independent of any

effects due to the presence of benzylpenicillin because erythromycin inhibited the biosynthesis of penicillinase when 2(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid was used as an inducer (Fig. 3). 2(2'-Carboxyphenyl)-benzoyl-6-aminopenicillanic acid is a gratuitous inducer (7) and has no detectable inhibitory activity against *S. aureus* 1206 at the concentration used in this experiment (unpublished data).

The implication of these experiments is that inhibition of penicillinase induction by erythromycin spares benzylpenicillin which, in turn, is able to inhibit growth once erythromycin resistance has been induced. The experiment summarized in Table 1 indicated that the antibiotic combination reduced the number of viable cells by more than 99%. Because benzylpenicillin but not erythromycin is bactericidal (8), the data support the notion that only one of the antibiotics (benzylpenicillin) is responsible for the observed inhibition of growth.

Clearly, the effectiveness of the benzylpenicillin-erythromycin combination is very much dependent on initial susceptibility to erythromycin. It might be expected, therefore, that constitutive resistance to erythromycin, as opposed to inducible resistance, would preclude susceptibility to the combination, because once resistance is established, penicillinase synthesis would no longer be sensitive to inhibition by erythromycin. *S. aureus* 1206 C4 is constitutive for erythromycin resistance but inducible for penicillinase. Unlike its parent, the minimal inhibitory concentration for the combination was the same as that for either antibiotic alone, namely, equal to or greater than 256 μg/ml. As expected, erythromycin had no measurable inhibitory effect on induced penicillinase biosynthesis in this strain (Table 2).

In an attempt to extend these observations, eight strains of *S. aureus* representing independent clinical isolates, each resistant to penicillin and erythromycin, were tested for their susceptibility to a combination of the two antibiotics (Table 3). All of these strains produce an inducible β-lactamase. Four strains were characterized as inducibly resistant to erythromycin, and, although they appeared to differ somewhat in degree, they were all susceptible to a combination of benzylpenicillin plus erythromycin. On the other hand, four strains demonstrated a constitutive phenotype for erythromycin resistance, and all were insusceptible to the combination.

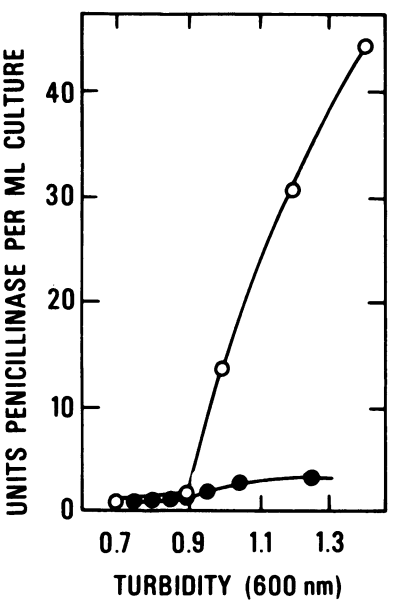

FIG. 3. *Erythromycin inhibition of 2(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid-induced penicillinase biosynthesis. Cells were grown, induced, and assayed for penicillinase as described in the legend to Fig. 1. 2(2'-Carboxyphenyl)-benzoyl-6-aminopenicillanic acid (3.5 μg/ml) was used to induce penicillinase in the absence (○) and presence (●) of 10 μg per ml. erythromycin.*

TABLE 1. *Bactericidal effect of the penicillin-erythromycin combination*[a]

Addition[b]	Colony-forming units/ml[c]	
	Before incubation	After incubation
None	1.4×10^5	2.0×10^8
Benzylpenicillin	1.4×10^5	1.6×10^8
Erythromycin	1.4×10^5	0.9×10^8
Combination	1.4×10^5	8.0×10^2

[a] Viability of *S. aureus* 1206 in tryptone-glucose broth was measured before and after 18 h of incubation in the presence and absence of antibiotics.

[b] Antibiotics were added at 50 μg/ml; the combination contained 50 μg of each antibiotic per ml.

[c] Cultures were diluted and plated on tryptone-glucose agar for isolated colonies.

TABLE 2. *Effect of erythromycin on induction of penicillinase by S. aureus 1206 C4*[a]

Erythromycin concn (μg/ml)	U of penicillinase/ml
0	2.0
1	2.2
10	2.1
100	2.0

[a] Penicillinase was induced for 60 min in the presence and absence of erythromycin and assayed as described in the legends to Fig. 1 and 2.

TABLE 3. *Susceptibility of clinically isolated S. aureus to the penicillin-erythromycin combination*

Strain[a]	Erythromycin resistance[b]	Minimal inhibitory conc[c] (μg/ml) of:		
		Benzylpenicillin	Erythromycin	Combination[d]
S10 orange	I	>256	>256	16
S10 cream	I	>256	>256	32
V41	I	>256	>256	4
3139	I	128	64	<1
3132	C	>256	>256	>256
Kagen 35	C	>256	>256	>256
Seattle 966	C	>256	>256	>256
Seattle 967	C	>256	>256	>256

[a] All strains produce an inducible β-lactamase.
[b] Phenotype of erythromycin resistance: I, inducible; C, constitutive.
[c] Incubation was for 24 h in tryptone-glucose broth.
[d] Equal concentrations of benzylpenicillin and erythromycin.

DISCUSSION

The antimicrobial activity of benzypenicillin against *S. aureus* is bactericidal and depends on inhibition of the transpeptidase-catalyzed cross-linking reaction in cell wall biosynthesis. In contrast, inhibition by erythromycin is bacteriostatic and is due to inhibition of polypeptide synthesis resulting from the action of this drug on the large ribosomal subunit. As would be expected, the mechanisms of resistance developed by staphylococci to these two antibiotics are also different. Penicillin resistance is due to production of a β-lactamase that hydrolyzes the antibiotic to an inactive form, whereas erythromycin resistance develops as a result of an alteration of the ribosome, which prevents ribosome binding by this antibiotic.

The studies reported here establish that the synergistic combination of benzylpenicillin and erythromycin against *S. aureus* 1206 is a result of inhibition of induced penicillinase biosynthesis by erythromycin. This, in effect, protects benzylpenicillin from enzymatic inactivation, subjecting the cells to the bactericidal effects of this antibiotic. Although both antibiotics are required for synergy, each acts independently of the other. There is no reason to suspect that the two antibiotics together behave in any manner unlike that expected when used separately against susceptible staphylococci. The combination is clearly an exception to the rule claiming that bacteriostatic drugs antagonize the activity of bactericidal antibiotics (8).

The dramatic response of *S. aureus* 1206 to the combination is owed to the fact that the induction of resistance to one antibiotic is sensitive to inhibition by the second. Although erythromycin interferes with development of resistance to benzylpenicillin, the latter antibiotic has no effect on the induction of erythromycin resistance. Subsequently, when the cells begin to divide in the presence of erythromycin, they are killed by benzylpenicillin. A mutant demonstrating constitutive erythromycin resistance was predictably insusceptible to the combination. This mutant is presumed to be a constitutive methylating strain (6) and, as such, would be insusceptible to the inhibition of penicillinase biosynthesis by erythromycin. The effectiveness of the two antibiotics together against *S. aureus* 1206 exemplifies how particular modes of resistance can facilitate a synergy. Based on the role played by erythromycin, any of the other inducing-type macrolide, lincosamide, and streptogram in B-type antibiotics (2) as well as erythromycin should be synergistic when combined with benzylpenicillin against this strain.

Previous reports (4, 5, 12, 14, 15) have described the use of penicillin and erythromycin combinations against strains of *S. aureus* resistant to both antibiotics individually. Herrell et al. (4) suggested the name "erythrocillin" to describe a combination of equal parts of propionyl erythromycin and penicillin that was effective against resistant *S. aureus* in both in vitro and clinical studies. Roberts et al. (14) concluded correctly that erythromycin was able to inhibit penicillinase and thereby prevent destruction of penicillin. However, the relationship between inducible erythromycin resistance and synergy in these strains was not recognized.

It is likely that the response of *S. aureus* 1206 and its constitutive mutant to the penicillin-erythromycin combination is typical of the behavior of most natural isolates of *S. aureus* resistant to these two antibiotics. Among eight clinical isolates examined in this study for susceptibility to the combination, only those inducibly resistant to erythromycin were susceptible. Those strains that were not inhibited by the combination had constitutive phenotypes. Demonstration of synergy by using the combination against strains of *S. aureus* that are susceptible to the two antibiotics when tested separately is apparently rare (4, 12). This fact, coupled with the data reported here, help, to explain the limited effectiveness of this synergistic combination.

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