ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1986, p. 877–882
0066-4804/86/120877-06$02.00/0

Vol. 30, No. 6

In Vitro Studies Simultaneously Examining Effect of Oxacillin on Uptake of Radiolabeled Streptomycin and on Associated Bacterial Lethality in *Staphylococcus aureus*

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Received 13 January 1986/Accepted 9 September 1986

We studied the effects of various concentrations of oxacillin on streptomycin uptake and killing for several strains of *Staphylococcus aureus*. When streptomycin was present in concentrations below the MIC, addition of oxacillin at concentrations greater than or equal to the MIC was associated with both significantly increased aminoglycoside uptake and killing. In contrast, when streptomycin was present in concentrations above the MIC, no increase of streptomycin uptake was noted with the addition of oxacillin, and killing was no greater than what would have been expected with a simply additive effect. Similar studies in a strain of *S. aureus* selected for high-level streptomycin resistance also demonstrated increased streptomycin uptake in the presence of concentrations of oxacillin above the MIC; however, killing was no greater than that seen with oxacillin alone. These studies provide data which are potentially important in designing a rational approach to clinical use of combination antibiotic therapy.

Combinations of β-lactam and aminoglycoside antibiotics are often used for synergism in the initial therapy of patients with infective endocarditis due to gram-positive pathogens (1, 17). The use of a combination of a β-lactam and an aminoglycoside for synergy is clearly important in the therapy of enterococcal infective endocarditis. Moellering and Weinburg have demonstrated that penicillin enhances the entry of otherwise sublethal concentrations of streptomycin (25), establishing that the mechanism of synergy in *Streptococcus faecalis* is similar to that demonstrated earlier in *Escherichia coli* by Plotz and Davis (27). Enhanced aminoglycoside uptake is commonly assumed to be the mechanism of potentiation with these antibiotics against both gram-positive and gram-negative organisms (7, 14, 15, 34). Studies in our laboratory examining the early bactericidal effects of β-lactams and aminoglycosides have, however, failed to demonstrate either synergistic killing or β-lactam-induced stimulation of aminoglycoside uptake in viridans group streptococci (M. A. El-Sokkary, F. D. Lowy, and M. H. Miller, Fed. Proc. **43**:371, 1984).

This potentially toxic therapeutic strategy is often recommended in the treatment of endocarditis due to *Staphylococcus aureus*, despite the fact that most studies fail to demonstrate improved survival with combination therapy (1, 17). The basis for these recommendations rests upon the more rapid clearance of bacteremia demonstrated clinically (17), as well as synergy as shown by in vitro time-kill studies and efficacy studies in an animal endocarditis model (22, 30).

Using a rabbit model of *S. aureus* endocarditis, we have shown that combination therapy with β-lactams and aminoglycosides at clinically relevant concentrations is associated with more rapid sterilization of infected valvular vegetations than is seen with either drug alone (22). However, even with aminoglycoside monotherapy, the majority of viable organisms persisting within valvular vegetations are phenotypically normal, gentamicin-susceptible organisms. Since reversion to the aminoglycoside-susceptible phenotype is an uncommon event both in vitro and in vivo, suppression of mutant overgrowth cannot be the sole explanation for the degree of potentiation observed (22).

Preliminary in vitro aminoglycoside uptake studies with nafcillin and [^{14}C]gentamicin at concentrations chosen to approximate the mean serum concentrations in the in vivo model (approximately 100- and 1.25-fold the MIC of nafcillin and gentamicin, respectively) showed equivocal stimulation of aminoglycoside uptake and no increased killing ($n = 5$). The mechanism of apparent in vivo synergism therefore remains an enigma.

Since an understanding of the mechanism(s) involved in drug potentiation both contributes to our knowledge of drug action and is important in designing rational approaches to combination therapy, we undertook these studies of the effects of β-lactams on the uptake of aminoglycosides and on the associated lethal effects in previously characterized, aminoglycoside-susceptible strains of *S. aureus*. Radiolabeled streptomycin was used, rather than gentamicin, because of the lack of availability of the latter reagent.

MATERIALS AND METHODS

Organisms and susceptibilities. The *S. aureus* strains used in these studies (SA86, SA121, SA121 *rpsL*, RN450, and RN450 *rpsL*) are previously characterized strains of diverse origins (21, 22). SA86 and SA121 are blood culture isolates from Montefiore Hospital Medical Center (17). RN450 is a plasmid-free, endonuclease-deficient strain (gift of Richard Novick), and RN450 *rpsL*, is a single-step, high-level streptomycin-resistant (MIC ≥ 2,500 μg/ml) mutant derived from RN450 by selection in broth containing 1,000 μg of streptomycin per ml. SA121 rpsL is a second such high-level streptomycin-resistant mutant selected from SA121.

The MICs of oxacillin and streptomycin for all strains were determined by standard tube dilution techniques, using

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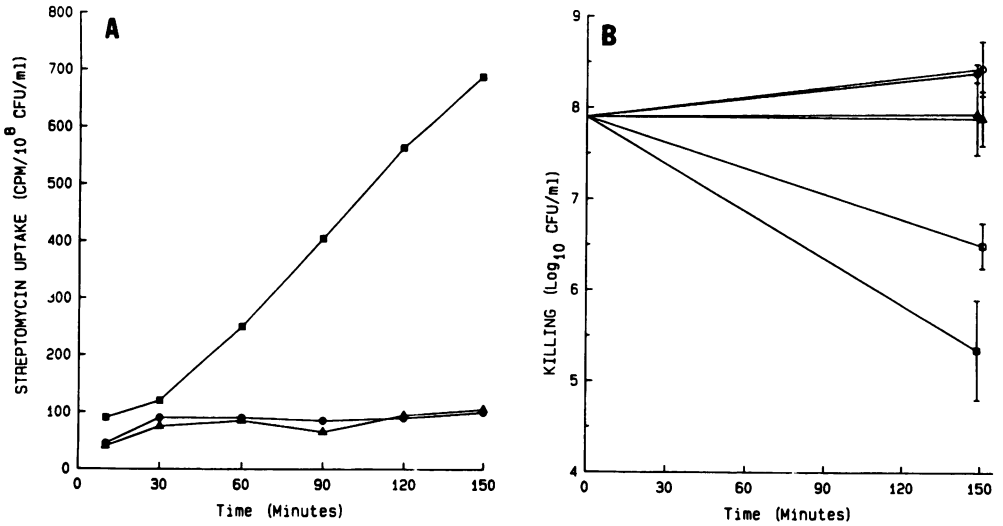

FIG. 1. (A) Effect of oxacillin concentrations above and below the MIC on streptomycin uptake by *S. aureus* SA86 at sub-MIC (0.2 μg/ml) of streptomycin. Symbols: ●, streptomycin alone; ▲, streptomycin plus 0.075 μg of oxacillin per ml; ■, streptomycin plus 2.0 μg of oxacillin per ml. (B) Corresponding killing. Open symbols represent killing with the same concentrations of oxacillin (△, 0.075 μg/ml; □, 2.0 μg/ml) in the absence of streptomycin; ○, control growth.

nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% yeast extract (NBYE) adjusted to pH 6.8 (8, 18) (Table 1). An inoculum of approximately 10^5 CFU/ml was used with standard antibiotic powder of reagent grade. Antibiotics were supplied by the following companies. Dihydrostreptomycin sesquisulfate (Sigma Chemical Co., St. Louis, Mo.) had a bioactivity of 866 μg/ml and was maintained in stock solution at 10,000 μg of bioactivity per ml. Oxacillin (gift of Beecham Laboratories, Bristol, Tenn.) had a bioactivity of 880 μg/ml and was maintained in stock solution at 6,400 μg of bioactivity per ml. Stock solutions were stored at −70°C before use. All MIC determinations were done in triplicate.

In vitro assays of antibiotic synergism. Abbreviated time-kill studies were performed in NBYE (pH adjusted to 6.8 with 1N NaOH or 1N HCl), from the same flasks as uptake studies (see below). Cultures of log-phase cells were incubated in a water bath at 37°C with gentle shaking as described previously (18). To determine cell viability, aliquots were removed at specified times after addition of antibiotics. To minimize the effects of antibiotic carry-over, samples were immediately diluted 1,000-fold in iced saline followed by serial twofold dilution. By using modification of a standard technique (18), we made pour plates of NBYE plus 1.5% agar (adjusted to pH 5.5 with 1 N HCl to minimize the effects of aminoglycoside carry-over) (8, 18, 21, 29). Statistical analysis was done by Student's *t* test.

Aminoglycoside uptake studies. Cells were grown overnight in NBYE (pH 6.8) at 37°C and were diluted 10^{-3} with fresh NBYE; growth at 37°C was monitored spectrophotometrically. Log-phase cells were divided into prewarmed flasks, and antibiotic(s) was added to the desired final concentration.

Cells were in log-phase growth at 10^7 to 10^8 CFU/ml during the experiments. Concentrations of antibiotics used included fractions and multiples of the MICs of each compound. For studies with subinhibitory concentrations, the antibiotic concentration chosen was the highest sub-MIC that demonstrated no significant killing at 6 h in standard time-kill studies when tested alone. This concentration was between 0.25 to 0.5 times the MIC for each strain tested. [^{3}H]dihydrostreptomycin sesquisulfate (Amersham Corp., Arlington Heights, Ill.) had a specific activity of 1.8 mCi/mmol and was mixed with unlabeled streptomycin stock solution to a final specific activity of 1 to 3 μCi/mg for experiments with 50 μg of streptomycin per mg. For experiments with low streptomycin concentrations (0.5 or 0.2 μg/ml), the final specific activity was 100 to 300 μCi/mg. At the indicated times, 5-ml samples were removed and immediately filtered through glass microfiber filters (Whatman GFC; Whatman, Inc., Clifton, N.J.) presoaked with streptomycin (1,000 μg/ml). Filters were washed with 5 ml of 3% NaCl and placed into counting vials, dried overnight, and counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.) after addition of 5 ml of toluene (Fisher Scientific Co., Pittsburgh, Pa.) with 0.5% 2,5-diphenyloxazole and 0.4% 1,4-bis-2-(5-phenyloxazole)benzene (Sigma). Absorbance was measured from each flask several times during the course of each experiment. Aminoglycoside uptake is expressed as counts of [^{3}H]streptomycin uptake per minute per 10^8 CFU as determined spectrophotometrically (see above) by using a predetermined correlation between absorbance and CFU (18). Uptake in nanograms per milliliter was calculated from the counts per minute by using the specific activity and a counter efficiency of 25%. Cell viability was determined simultaneously from the same cultures, as described above.

RESULTS

Effect of oxacillin on streptomycin uptake and killing in susceptible *S. aureus* at streptomycin concentrations below the MIC. Figure 1A demonstrates the effect of oxacillin on the uptake of radiolabeled streptomycin in *S. aureus* 86. Figure 1B shows the associated bactericidal effects of these antibiotics used alone and in combination. The MICs of both oxacillin and streptomycin are shown in Table 1. At streptomycin concentrations below the MIC (i. e., 0.5 times the MIC) there was little uptake of [^{3}H]streptomycin and no loss of viability in the absence of oxacillin. When oxacillin was used at concentration of 0.25 MIC, there was no stimulation of streptomycin uptake, and the effect on cell viability was

TABLE 1. Streptomycin and oxacillin MICs for *S. aureus*

Strain	MIC (μg/ml)	
	Streptomycin	Oxacillin
RN450	2.0	0.5
SA86	0.4	0.25
RN450 *rpsL*	≥2,500	0.5
SA121 *rpsL*	≥2,500	0.25

no greater than that seen with oxacillin alone. In contrast, when cells were exposed to oxacillin at concentrations which were eight times the MIC, after a delay of approximately 30 min there was a marked enhancement of streptomycin uptake. Moreover, this increased streptomycin uptake was associated with a significantly greater loss of viability at 2.5 h than was seen with either drug alone ($P < 0.05$). Figures 1A and B show the mean of three experiments. The calculated oxacillin-stimulated streptomycin uptake at 150 min is approximately 10 ng/10^8 CFU. Similar results were obtained by using comparable concentrations of oxacillin and streptomycin in a second *S. aureus* strain, RN450 (data not shown). In RN450, however, there was slight stimulation of streptomycin uptake (0.25 times the MIC) with oxacillin at 0.5 times the MIC. Preexposure of cells to oxacillin at eightfold the MIC for 30 min, or continued incubation for 3 to 4 h in cells where antibiotics were added simultaneously was associated with a >2 $\log_{10}$ increase in killing effect of the combination as compared with either drug alone, thus fulfilling standard criteria for bactericidal synergism (26). No differences in growth as measured by absorbance were noted between cultures treated with oxacillin alone versus those treated with oxacillin plus streptomycin over the time course of these experiments (data not shown).

Dose-response curves showing uptake of the same sub-MIC of streptomycin by *S. aureus* RN450 exposed to concentrations of oxacillin ranging from 0.5- to 16-fold the MIC (0.25 through 8 μg/ml) demonstrated that the observed stimulation of streptomycin uptake occurred in an oxacillin dose-dependent fashion. Preexposure of cells to the same concentrations of oxacillin for 30 min before the addition of streptomycin abolished the delay in increased streptomycin uptake which occurred when drugs were added simultaneously (data not shown).

Effect of oxacillin on streptomycin uptake at streptomycin concentrations above the MIC. When *S. aureus* RN450 was exposed to streptomycin at 20 and 50 μg/ml (MIC = 2 μg/ml), after a delay of 30 min uptake was seen at both concentrations and was proportional to streptomycin concentration, as has been demonstrated for gentamicin (18, 21). Simultaneous killing studies demonstrated a decrease in CFU per milliliter beginning at about 30 min, which was approximately 3 $\log_{10}$ at 150 min at both streptomycin concentrations.

Uptake was noted with [^{3}H]streptomycin alone (50 μg/ml) at the earliest sample point and continued at a decreasing rate over 150 min (total uptake was approximately 900 ng/10^8 CFU) (Fig. 2A). Addition of oxacillin either above (8.0 μg/ml) or below (0.25 μg/ml) the MIC was associated with no increase in observed streptomycin uptake. Consistently, the loss of viability seen with these combinations of oxacillin and streptomycin was no greater than that which would be expected from an additive effect (Fig. 2B). The data in Fig. 2A and B represent mean values of two experiments. Identical results were obtained by using [^{3}H]streptomycin at concentrations of 40- and 100-fold the MIC and oxacillin 0.25- and 10-fold the MIC in *S. aureus* 86 (data not shown).

Effect of oxacillin on [^{3}H]streptomycin uptake and killing in *S. aureus* mutants selected for a high-level resistance to streptomycin. The effects of oxacillin on [^{3}H]streptomycin uptake and killing in a single-step, high-level resistant mutant of RN450 (RN450 *rpsL*) are shown in Fig. 3. There was little uptake of [^{3}H]streptomycin and no killing with streptomycin alone at a concentration of 50 μg/ml (MIC >2,500 μg/ml). The addition of oxacillin at concentrations up to 0.25 times the MIC (0.25 μg/ml) had little effect on [^{3}H]streptomycin uptake or killing. Addition of oxacillin at 16 times the MIC (8 μg/ml) was associated with a marked stimulation of streptomycin uptake similar to that seen in the streptomycin-susceptible strain. In contrast to the streptomycin-susceptible strain, however, this increased uptake

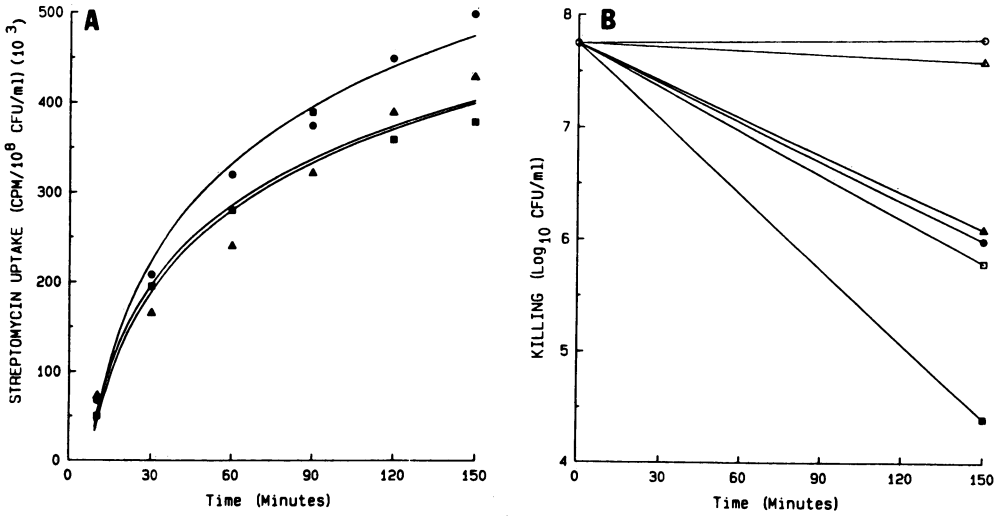

FIG. 2. (A) Effect of oxacillin concentrations above and below the MIC on streptomycin uptake by RN450. Streptomycin was present in concentrations above the MIC in all flasks (50 μg/ml). Data are for streptomycin alone (●) and with 0.25 (▲) and 8.0 (■) μg of oxacillin per ml. (B) Corresponding killing. Open symbols represent killing with the same two concentrations of oxacillin without streptomycin (△, □); ○, control growth.

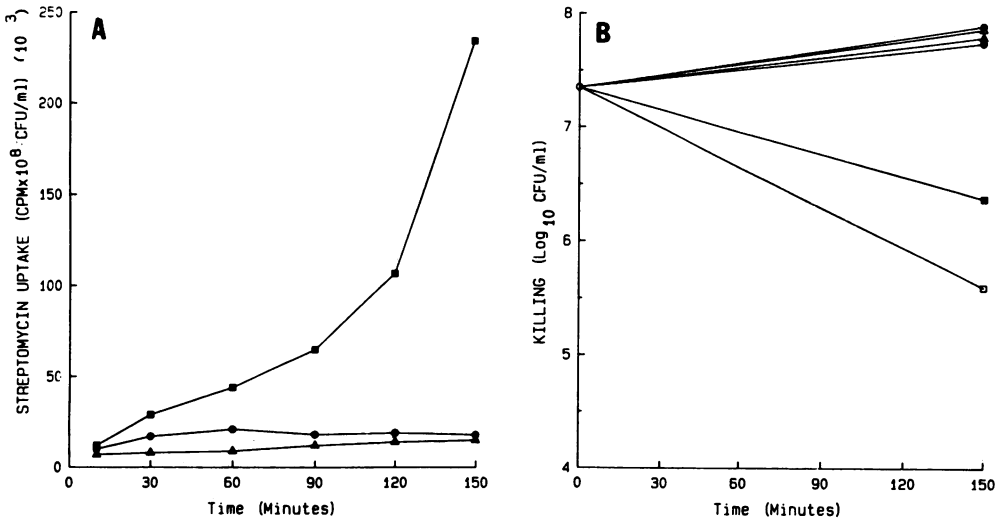

FIG. 3. (A) Effect of oxacillin concentrations above and below the MIC on uptake of 50 μg of streptomycin per ml in RN450 *rpsL* (MIC >2,500 μg/ml). Uptakes of streptomycin alone (●) and in the presence of 0.125 (▲) and 8.0 (■) μg of oxacillin per ml are shown. (B) Corresponding killing. Open symbols represent killing with oxacillin alone (△, □); ○, control growth.

was not associated with an enhanced bactericidal effect beyond that seen with oxacillin alone. The apparent decreased killing with the combination as compared with oxacillin alone is not significant, and this difference was not seen with higher concentrations of oxacillin or at later time points or when cells were preincubated with oxacillin. Data shown represent the mean of two experiments. Identical results were obtained in another one-step high-level streptomycin-resistant mutant, SA121 *rpsL* (data not shown).

DISCUSSION

Early studies by Plotz and Davis (27) demonstrate in a streptomycin-susceptible strain of *E. coli* that pretreatment with penicillin is associated with both increased streptomycin uptake and increased killing. Experiments by Moellering et al. extend these observations to enterococci; in *Streptococcus faecalis*, penicillin exposure results in enhanced aminoglycoside uptake and killing in streptomycin-susceptible strains (25, 26). Based on these observations, β-lactam-induced augmentation of aminoglycoside uptake has been commonly invoked as the mechanism of the synergistic killing of *S. aureus* observed in in vitro time-kill studies and in animal models (22). Moreover, these same data have been used to support antibiotic combination therapy in less well-defined clinical situations, including the use of penicillin and streptomycin (or gentamicin) to treat endocarditis due to viridans group streptococci (24).

Studies examining the effect of nafcillin and gentamicin, alone or in combination, on the viability of organisms in valvular vegetations of rabbits with *S. aureus* endocarditis (21, 22, 30) suggest other potential mechanisms for such synergy including inhibition by nafcillin of regrowth of aminoglycoside-resistant mutants. The inhibition of emergence of such aminoglycoside-resistant mutants appears to be one factor in drug potentiation seen both in vivo and in vitro. However, alternative mechanisms of potentiation are needed to explain the markedly enhanced sterilization of valvular vegetations seen in vivo because (i) the mutant strain isolated during those studies has a very low frequency of reversion to phenotypically wild-type organisms, and (ii) the organisms remaining viable in valvular vegetations of the animals treated with gentamicin alone are largely of the parent phenotype (22).

The present studies showed that in streptomycin-susceptible *S. aureus* exposed to streptomycin at concentrations below the MIC, the addition of the β-lactam oxacillin (at concentrations above the MIC of that drug) was associated with a marked increase in the uptake of [^{3}H]streptomycin. This increased uptake occurred after a lag of about 30 min. The lag was eliminated, and total uptake slightly increased, by pretreatment of cultures with oxacillin for 30 min before the addition of [^{3}H]streptomycin. This increased uptake of streptomycin was associated with greater killing than was seen with oxacillin alone, or than would be expected by a simple additive effect of the two drugs. Moreover, while the criteria for bactericidal synergism are arbitrary (23), continued incubation of cells to which the antibiotics were added simultaneously for 3 to 4 h, or preexposure to oxacillin for 30 min, was associated with a >2 $\log_{10}$ increase in bactericidal effect with the combination, thus fulfilling the criteria for synergy as commonly defined (26). When oxacillin was used at concentrations of 0.25 times the MIC or less, it caused little or no stimulation of streptomycin uptake at 0.5 times the MIC, and there was no increased killing.

When these studies were performed at streptomycin concentrations above the streptomycin MIC, we were unable to demonstrate any increase in streptomycin uptake with the addition of either low or high concentrations of oxacillin. The amount of killing observed, although greater than that observed for each drug alone, was approximately that which would be expected in the case of a simple additive effect. Studies of the effect of oxacillin on the uptake of [^{3}H]streptomycin at sub-MIC in single-step streptomycin-resistant mutants (MIC ≥ 2,500 μg/ml) of *S. aureus* also demonstrated stimulation of [^{3}H]streptomycin uptake. In these strains, however, the killing observed was no greater than that seen with oxacillin alone. These observations in resistant *S. aureus* provide evidence that, whatever the mechanism of β-lactam stimulation of aminoglycoside uptake, it is independent of aminoglycoside-ribosome binding, and likely represents some primary alteration of the cell wall or cell membrane. Furthermore, they are consistent with

current concepts of aminoglycoside action that ribosomal binding is necessary for lethal effect (5, 11, 12). Although there are well-recognized differences between aminoglycosides in terms of their interaction with the ribosome, previously published work comparing uptake of streptomycin and gentamicin in SA86 (8), the occurrence of augmented uptake in *rpsL* mutants, and the similarity of the present results to preliminary studies with [^{14}C]gentamicin, strongly suggest that the observations presented here would be generally applicable to other more commonly used aminoglycosides.

Our inability to demonstrate increased aminoglycoside uptake by addition of oxacillin, when the [^{3}H]streptomycin concentration was above the MIC, is more difficult to interpret. For susceptible organisms exposed to aminoglycosides at concentrations above the MIC, both the rate of uptake and total drug accumulation are directly proportional to the aminoglycoside concentration (8, 12, 18, 21; also see above). Despite the concentration dependence of drug accumulation and a correlation between amount of uptake and early killing, there is no difference in the amount of killing seen after a period of hours (8; see above). It is likely that although susceptible organisms may be made to accumulate greater amounts of drug at a faster rate, once the minimal amount of drug needed to achieve maximal lethal effect has gained access to its target, such augmented uptake is superfluous in regard to late killing.

It is also possible that under conditions where each drug alone is present in concentrations sufficient to cause significant lethal effect, the rapid loss of viable organisms capable of incorporating labeled aminoglycoside would mask any increased uptake that might occur in the cells that are still viable. Alternatively, at concentrations above the MIC, streptomycin itself, like other inhibitors of cell growth, might block the penicillin effect responsible for the augmentation fo [^{3}H]streptomycin uptake. It is not possible from the available data to distinguish between these possibilities.

Finally, the mechanism by which a β-lactam antibiotic or other cell-wall-active agent enhances the uptake of an aminoglycoside in a gram-positive organism is a matter of conjecture. Gram-positive cell walls are composed of a thick layer of cross-linked peptidoglycan and teichoic acid (9, 16, 32, 33). The physical structure of these cell walls should allow free movement of molecules of the size of these antibiotics and therefore should not function as a barrier for either β-lactams or aminoglycosides. Teichoic acids, however, are negatively charged polymers which bind divalent cations as well as polycationic aminoglycosides (2–4). Experiments in streptomycin- and neomycin-producing *Streptomyces* spp. have shown ionic binding of streptomycin to the negatively charged teichoic acid groups of the cell wall (31). The viridans streptococci, *S. sanguis* and *S. mutans*, are known to contain significantly less teichoic acid polymer than do staphylococci (10, 32). Our data indicating that β-lactams increase aminoglycoside uptake by *S. aureus* (see above) and *Streptococcus faecalis*, but not in viridans group streptococci (El-Sokkary, et al. Fed. Proc., 1984), suggest a mechanistic explanation for these observations. According to this model, at relatively low streptomycin concentrations (below the MIC), binding of streptomycin to teichoic acid might prevent access of molecules to the plasma membrane carrier proteins and prevent subsequent streptomycin transport into the cell. Since β-lactam antibiotics cause release of teichoic acid and peptidoglycan into growth media (13, 28), these drugs would likely diminish this physiologic ion-exchange resin, allowing free access of drug to the membrane. At high aminoglycoside concentrations, all of the anionic binding sites would likely be saturated, allowing drug molecules to reach the cell membrane; hence, addition of oxacillin and the resulting decrease of potential binding sites would have no discernable effect in terms of drug entry or killing.

These studies show that, in the presence of concentrations of streptomycin which are a small fraction of achievable serum concentrations or MIC cutoffs used in susceptibility tests, addition of oxacillin in concentrations above the MIC is associated with a potentiating effect in susceptible *S. aureus*, both mechanistically (in terms of increased streptomycin uptake) and functionally (in terms of synergistic killing). It is important to point out that although neither synergy nor enhanced uptake was seen when concentrations of drugs comparable to those achievable in sera of infected patients were used (streptomycin concentrations > MIC), it is likely that functionally subinhibitory aminoglycoside concentrations occur within valvular vegetations due to poor local penetration of drug, or locally decreased pH or redox potential—conditions which have been shown to adversely affect both aminoglycoside uptake and lethal effect (5, 6, 8, 11, 19, 20).

These studies provide additional in vitro evidence that, for serious staphylococcal infections such as endocarditis, the combined use of β-lactams and aminoglycosides may be rational on the basis of more than one mechanism. These mechanisms include augmentation of aminoglycoside uptake, as well as inhibition of regrowth of aminoglycoside-resistant mutants.

ACKNOWLEDGMENTS

We thank Joyce Williams for her valuable technical assistance and Marie Torlone for her secretarial support.

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