ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1983, p. 440–442
0066-4804/83/090440-03$02.00/0

Vol. 24, No. 3

Effect of *N*, *N'*-Dicyclohexylcarbodiimide and Nigericin on *Staphylococcus aureus* Susceptibility to Gentamicin

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Received 15 April 1983/Accepted 30 June 1983

The abilities of the H^+-ATPase inhibitor *N*, *N'*-dicyclohexylcarbodiimide and the antibiotic ionophore nigericin to enhance the bactericidal effect of subinhibitory concentrations of gentamicin in two strains of *Staphylococcus aureus* were studied. Each compound significantly increased both gentamicin uptake and killing. The use of agents which alter the plasma membrane energy state is a novel approach to enhance the activity of conventional antibiotics.

Antibiotics such as aminoglycosides must traverse the plasma membrane before interacting with their active sites on bacterial ribosomes. Recent studies of antibiotic uptake in our laboratory suggest that the uptake of the cationic aminoglycoside gentamicin in *Staphylococcus aureus* is regulated by the electrical potential ($\Delta\psi$) (3, 4, 6), as predicted by chemiosmotic theory (8). The clinically relevant synergy of cell wall-active antibiotics and aminoglycosides against enterococci has been shown by Moellering and Weinberg to involve enhanced aminoglycoside cellular entry (9). Our studies suggest an alternate approach to enhancing the entry of aminoglycosides by using agents which alter the plasma membrane energy state. The compounds nigericin and *N*, *N'*-dicyclohexylcarbodiimide (DCCD) are agents which cause specific alterations in plasma membrane energization. Nigericin increases the uptake of subinhibitory concentrations of aminoglycosides in *S. aureus* at acid pH and under anaerobiosis (3, 4, 6), conditions often present in the microenvironment of infected tissue. The present studies were undertaken to determine whether this enhanced uptake is associated with an increased bactericidal effect in gentamicin-susceptible *S. aureus*.

Two previously characterized methicillin- and gentamicin-susceptible strains of *S. aureus* (SA 121 [6] and SA Seattle [ATCC 25923]) were studied. The minimal inhibitory concentrations of gentamicin were determined (7) for each strain in nutrient broth with 0.1% yeast extract and were 0.4 and 0.1 μg/ml, respectively, at pH 6.8 and 6.2 μg/ml for both strains at pH 5.0. The uptake of radiolabeled gentamicin was determined by membrane filtration as previously described (6). Nigericin and DCCD were added at various times and final concentrations (see Fig. 1). Cell viability (in CFU per milliliter) was determined by a standard pour plate technique (7). [^{3}H]gentamicin (Amersham Corp., Arlington Heights, Ill.) was mixed with standard gentamicin powder (Shering Corp., Bloomfield, N.J.) to a final specific activity of 5 μCi/mg. DCCD was obtained from Sigma Chemical Co., St. Louis, Mo., and nigericin was a gift from J. Wesley of Hoffmann-LaRoche Inc., Nutley, N.J.

Figure 1 shows both the bactericidal effect and the uptake of gentamicin at pH 6.8 at one-fourth the minimal inhibitory concentration of gentamicin for SA Seattle and SA 121, with and without 20 μM DCCD. The standard minimal inhibitory concentration of DCCD under these conditions was greater than 500 μM, and there was no killing with 20 μM DCCD. In the absence of DCCD, there was little gentamicin uptake and cells continued to replicate. In contrast, when cells were exposed to 20 μM DCCD, gentamicin uptake occurred after a lag of 10 min and was associated with a diminution in CFU per milliliter. When these studies were repeated under anaerobic conditions or in the presence of 0.2 mM KCN, DCCD showed no stimulation of gentamicin uptake and no bactericidal effect (data not shown).

Figure 2 shows both the [^{3}H]gentamicin uptake and the bactericidal effect of a subinhibitory concentration of gentamicin at pH 5.0 in SA Seattle and SA 121, with and without 0.5 μM nigericin. This concentration of nigericin was associated with no killing. In the absence of nigericin, there was little drug uptake and no bactericidal effect. In contrast, when nigericin

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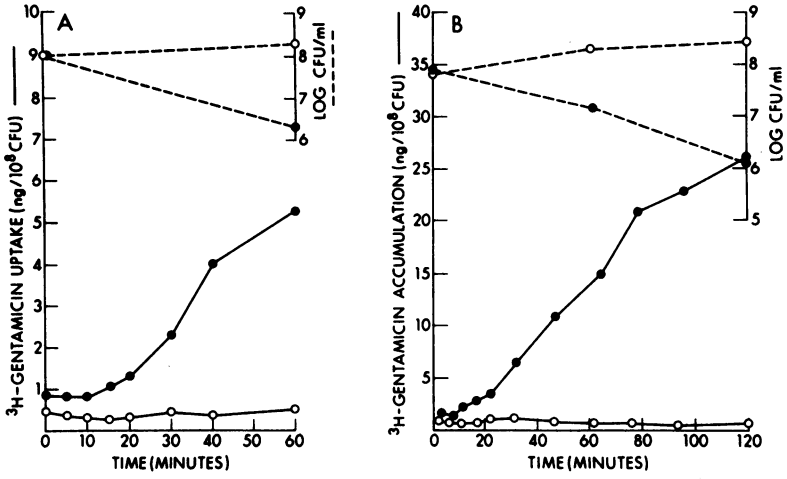

FIG. 1. [^{3}H]gentamicin uptake (—) and killing (--) in the presence (●) and absence (○) of 20 μM DCCD. Both DCCD and gentamicin were added at 0 min. The gentamicin concentrations studied were 0.025 μg/ml for SA Seattle (A) and 0.1 μg/ml for SA 121 (B), one-fourth of the minimal inhibitory concentration of gentamicin for each strain at pH 6.8.

was added, there was immediate gentamicin uptake and rapid killing.

These studies demonstrate that gentamicin uptake induced by DCCD and nigericin is associated with enhanced killing in gentamicin-susceptible strains of *S. aureus*. Previous studies of SA 86 have demonstrated that nigericin increases the magnitude of $\Delta\psi$ (3). Similarly, as predicted previously (6), 20 μM DCCD increases the magnitude of $\Delta\psi$ by ca. −20 mV (unpublished data).

DCCD is a toxic carboxyl reagent which reacts with the F_0 portion (channel) of the proton-translocating ATPase found in the cytoplasmic membrane of procaryotes and blocks H^+ translocation (2). The inability of DCCD to stimulate gentamicin uptake in a respiratory mutant of *S. aureus* (6) in the presence of KCN and under anaerobic growth conditions reflects the requirement for a functioning respiratory chain to generate an adequate $\Delta\psi$ for aminoglycoside uptake.

Nigericin, like vancomycin, is an antibiotic active against gram-positive organisms and is

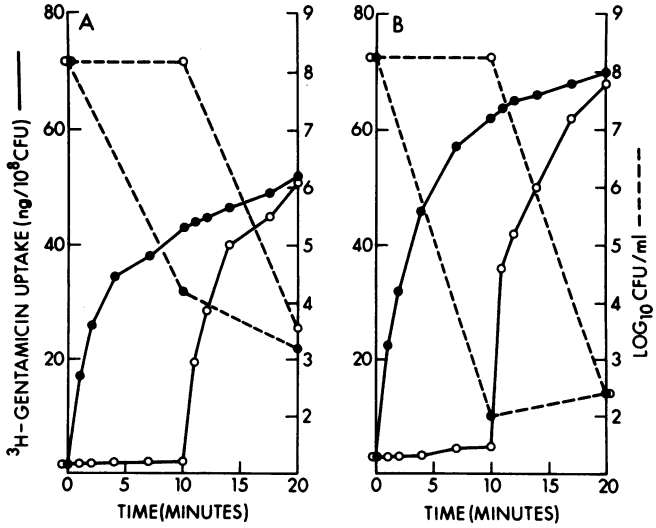

FIG. 2. [^{3}H]gentamicin uptake (—) and killing (--) in the presence and absence of 0.5 μM nigericin in SA Seattle (A) and SA 121 (B). Gentamicin concentrations studied were 1.0 and 5.0 μg/ml, respectively, and nigericin (0.5 μM) was added at 0 min (●) and 10 min (○).

derived from *Streptomyces* spp. Unlike DCCD, which is exceedingly toxic, the concentration of nigericin required to stimulate gentamicin uptake in vitro is a small fraction of the 50% lethal dose for mice (13). Nigericin catalyzes the electroneutral exchange of K^+ for H^+ which, at acid pH, is associated with a fall in ΔpH and an increase in $\Delta\psi$ in SA 86 (3).

Our studies of *S. aureus* describe a strategy to increase aminoglycoside uptake by manipulating the bioenergetics of the cytoplasmic membrane. The activity of other antibiotics may also be dependent on the proton motive force and subject to similar manipulations of the membrane energy state. Tetracycline, for example, requires an electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$) for transport (5). Moreover, it is likely that chemiosmotic forces affect the activity of antibiotics which do not require transport into cells. Recent studies show that the lethal action of β-lactam antibiotics requires both the integrity of extracytoplasmic binding proteins (12) and protein channels (porins) (10) which permit the entry of hydrophilic antibiotics into the periplasm of gram-negative bacilli. The processing and functional integrity of such proteins may require the ability of cells to generate $\Delta\tilde{\mu}_{H^+}$ (1, 11). These findings suggest that manipulations of the membrane energy state may provide a clinically useful approach to enhancing the activity of different classes of antibiotic compounds.

L.J.M. and E.S.E. were supported by Public Health Service training grant T01-A1-00405 from the National Institute of Allergy and Infectious Diseases.

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