Effects of Production of Abnormal Proteins on the Rate of Killing of Escherichia coli by Streptomycin

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The role of abnormal membrane proteins in modulating the rate of killing by streptomycin was investigated. Davis et al. (B. D. Davis, L. Chen, and P. T. Tai, Proc. Natl. Acad. Sci. USA 83:6164–6168, 1986) have proposed that misread membrane proteins created by the action of streptomycin on translating ribosomes cause the formation of nonspecific membrane channels which allow increased uptake of the antibiotic and contribute to its bactericidal action. Pretreatment of Escherichia coli with a low concentration of puromycin enhanced the rate of killing by streptomycin. The effect of the pretreatment with puromycin was transient, since approximately normal rates of killing by streptomycin were restored after 30 min of incubation in antibiotic-free medium. This time period correlates with the time required to degrade labile polypeptides in puromycin-treated cells. The induction of a specific abnormal malE-lacZ fusion protein, which is capable of disrupting the normal membrane protein secretion process, also increased the rate of killing by streptomycin.

The precise mode of action of the antibiotic streptomycin has been actively investigated for many years (for reviews, see references 6, 13, 14, and 26). Following a rapid, energy-independent, ionic interaction of streptomycin with cell surface components, there is an initial, slow rate of uptake of streptomycin, which has been called energy-dependent phase I (EDPI) and which requires establishment of an electrical potential across the membrane (3, 4). The onset of a second, more rapid phase of uptake, energy-dependent phase II (EDPII), is coincident with the initial inhibition of protein synthesis (4) and is suggested to occur at about the time of the lethal event (13, 14).

The exact nature of the membrane-associated carriers involved in the respiration-dependent uptake or even whether the increased cell association of aminoglycosides during EDPI involves intracellular accumulation or increased binding to the cytoplasmic membrane (21) is unknown. The rapid uptake found during EDPII is kinetically irreversible (20, 21) and does not occur in streptomycin-resistant cells (3, 4) or when protein synthesis is blocked by inhibitors (27, 28). Davis and co-workers (6–8) have proposed that abnormal membrane proteins play an essential role in the uptake and bactericidal action of aminoglycosides. Their model suggests that during EDPI an initial, low level of streptomycin enters the cell, interacts with elongating ribosomes, and causes a small degree of misreading (5). Some of the misread proteins are incorporated into the membrane, where their poor fit creates nonspecific channels which allow an additional influx of the antibiotic. An accelerating cascade of increased misreading and increased leakiness then occurs which culminates in the onset of EDPII. The intracellular antibiotic concentration then reaches a concentration that blocks initiating ribosomes and leads to the lethal event.

The model of Davis (6) is consistent with the observation that treatment with other agents that cause the formation of abnormal proteins leads to increased streptomycin uptake (16, 27, 28). For example, puromycin, which causes premature release of polypeptide chains, can cause a more rapid uptake of streptomycin in either wild-type (rpsL+) or streptomycin-resistant (rpsL) strains (16). Davis and co-workers (6–8) have suggested that the insertion of truncated puromycyl peptides into the membrane enhances the uptake of streptomycin. Another explanation for these data was offered by Hurwitz et al. (16), who suggested that the breakdown of polysomes induced by puromycin increases potential high-affinity streptomycin-binding sites on runoff ribosomes within the cell.

In the studies reported here, we examined the role of abnormal proteins in the bactericidal action of streptomycin. We investigated the influence of the pretreatment of cells with puromycin on the subsequent rate of killing by streptomycin to separate the effects of production of abnormal polypeptides by puromycin from its effects at the level of the ribosome. In addition, we examined whether the induction of specific abnormal membrane fusion proteins enhances the rate of killing by streptomycin.

MATERIALS AND METHODS

Bacterial strains. Sources and characteristics of the Escherichia coli strains used in this study are given in Table 1.

Media. Basal salts (BS) medium (22) was used for the determination of rates of killing by streptomycin and rates of protein degradation. The BS medium contained glucose (final concentration, 0.4%), thiamine (final concentration, 10 μg/ml), and yeast extract (final concentration, 0.02%). Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.) were used for viability determinations.

Protein degradation. E. coli KD100 was grown to the early exponential phase in BS medium without yeast extract. The culture was divided into two aliquots. One culture was treated with puromycin (50 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 15 min. Both the co-
trol culture and the puromycin-treated culture were labeled for 15 min with L-3,4,5-[^3H]leucine (1,243.5 Ci/mmol; Du-
port, NEN Research Products, Boston, Mass.) at a final concentration of 2 μCi/ml. The cultures were harvested by filtration on membranes (Millipore Corp., Bedford, Mass.) and washed two times with BS medium supplemented with excess unlabeled leucine (final concentration, 300 μg/ml). Cultures were suspended in BS medium with excess leucine; and four 0.1-ml portions were removed at various times and placed in Microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) containing 10 μl of 100% trichloroacetic acid (TCA) and 10 μl of a stock solution containing 10 μg of bovine serum albumin per ml. The TCA-soluble radioactivity in 50-μl samples from each tube was determined by using rpi 3470 complete cocktail. The radioactivity in the time zero TCA-precipitable material was determined following solubilization of the pellet with 100 μl of Soluene (diluted 9:1 with 
H2O).

Determination of susceptibilities of bacterial strains to streptomy-
cin. MICs were determined by using a modification of the spot test method described by Humbert and Altendorf (15).

For the time-kill studies we used early-exponential-phase cultures (optical density at 600 nm, approximately 0.1 to 0.15). The cultures were left untreated or were exposed to various treatments which would lead to the production of abnormal proteins. The cultures were harvested by centrifuga- tion and suspended in fresh, prewarmed medium. Streptomy-
cin (Sigma Chemical Co., St. Louis, Mo.) was added at either 50 or 100 μg/ml, and the cultures were incubated with aeration at 37°C. Viable counts were determined by plating the cultures in triplicate onto TSA.

RESULTS

Effects of pretreatment with puromycin on subsequent streptomy-
cin sensitivity. Simultaneous treatment with moderate concentrations of puromycin accelerates streptomycin uptake (16) and killing (27, 28). Puromycin treatment leads to the production of abnormal, prematurely released polypep-
tides and increases the rate of polysome breakdown to monosomes (16). To examine whether the abnormal proteins

produced during puromycin treatment were the critical fac-
tor in the increased rate of killing by streptomycin, we pre-
treated strain KD100 with puromycin (50 μg/ml) for 30 min. This pretreatment had no effect on cell viability. The culture was removed from puromycin-containing medium prior to the addition of streptomycin so that any effect on the rate of killing could not be ascribed to a synergistic effect of the antibiotics at the level of the ribosome. The rate of killing by streptomycin of KD100 pretreated with puromycin was compared with that in control cultures without puromycin pretreatment (Fig. 1). The pretreatment increased the rate of killing significantly; the time for culture viability to decrease 98% was 90 min for the puromycin-treated culture and 360 min for the control culture. It should be noted that although puromycin pretreatment increased the rate of killing by streptomycin, it did not decrease the MICs. Complete inhi-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or derivation</th>
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<tbody>
<tr>
<td>JW375</td>
<td>F+ supE42 zhc-511::Tn10 λ− (zhc-511::Tn10 contransduces approx 47% with rpsL+)</td>
<td>B. Bachmann</td>
</tr>
<tr>
<td>MC4100</td>
<td>F+ araD139 Δ(argF-lac)U169 rpsL150 relA fbbB5301 dele1 ptsF25 rbsR thiA</td>
<td>J. Beckwith</td>
</tr>
<tr>
<td>MM18</td>
<td>MC4100 (malE-lacZ) hyb 72-47</td>
<td>J. Beckwith</td>
</tr>
<tr>
<td>KD100</td>
<td>MC4100 rpsL+ (zhc-511::Tn10)</td>
<td>P1 transductant of MC4100 from JW375</td>
</tr>
<tr>
<td>MW400</td>
<td>MM18 rpsL+ (zhc-511::Tn10)</td>
<td>P1 transductant of MM18 from JW375</td>
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<td>DHB502</td>
<td>F+ (lacPpro+) araD139 Δ(aara-leu)7697 ΔlacX74 ΔphoAPuv11 phoR ΔmalF3 galk galK thi rpsL pcnB zad::Tn10 containing plasmid pSX4.29c (pBR322 bla and ori, malF-phoA fusion [fused at base 1417 of malF] under control of tac promoter, Tc′ inactivated)</td>
<td>D. Boyd</td>
</tr>
<tr>
<td>DHB510</td>
<td>Same as DHB502 containing plasmid pSX102 (pBR322 bla and ori, malF-phoA fusion [fused at base 826 of malF] under control of tac promoter, Tc′ inactivated)</td>
<td>D. Boyd</td>
</tr>
<tr>
<td>MW512</td>
<td>DHB502 lacking zad::Tn10; rpsL+ zhc-511::Tn10</td>
<td>P1 transductant of Tet′ derivative of DHB502 from JW375</td>
</tr>
<tr>
<td>MW520</td>
<td>DHB510 lacking zad::Tn10; rpsL+ zhc-511::Tn10</td>
<td>P1 transductant of Tet′ derivative of DHB510 from JW375</td>
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<td>MW601</td>
<td>MW601 cured of pSX102 (Amp+)</td>
<td>Spontaneous plasmid loss</td>
</tr>
<tr>
<td>MW611</td>
<td>MW601 containing plasmid pHS17 (pBR322 bla and ori, malF-malG; under control of tac promoter, Tc′ inactivated)</td>
<td>MW601 transformed by plasmid pHS17 (from H. Shuman)</td>
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FIG. 1. Effect of pretreatment with puromycin on the rate of killing of strain KD100 by streptomycin. An early-exponential-phase culture was divided in half, and one portion was exposed to puromycin (50 μg/ml) for 30 min. Both cultures were centrifuged and suspended in BS medium containing streptomycin (100 μg/ml). The changes in viable organisms per milliliter as a function of time were determined. O, Control culture; ⋄, puromycin-treated culture. Each datum point is the average of triplicate plate counts from four to eight separate experiments.
FIG. 2. Degradation of proteins in KD100 cultures labeled in the presence or absence of puromycin. A mid-exponential-phase culture was divided in half and pulse-labeled for 15 min with \(^{3}H\)leucine in the presence or absence of puromycin. The degradation of the labeled proteins in both cultures was determined. ○, Control culture; ●, puromycin-treated culture.

bition of growth occurred at 5 \(\mu g/ml\) for both cultures, as measured by growth for 16 to 18 h on agar plates. Active protein synthesis was also required for streptomycin killing in either puromycin-pretreated or control cultures. Addition of chloramphenicol (20 \(\mu g/ml\)) prevented the further loss of viability whenever it was added to the streptomycin-treated cultures (data not shown).

Protein degradation following puromycin treatment. Puromycin peptides have abnormal structures and are substrates for protein degradation (10, 11). Figure 2 shows the rates of protein degradation in cultures of strain KD100 which were labeled in the presence or absence of puromycin. The initial rate of degradation of proteins labeled during puromycin treatment was approximately four times the rate of degradation of normal polypeptides in the control culture. After 45 min, most of the labile polypeptides were degraded, so that the rates of degradation in both cultures between 45 and 90 min were similar (1.6% in the control culture and 2.0% in the puromycin-treated culture). To examine whether removal of the labile polypeptides by protein degradation restored the normal kinetics for streptomycin uptake and killing, cultures were pretreated with puromycin and then either exposed immediately to streptomycin or allowed to recover for 30 min in medium lacking puromycin prior to exposure to streptomycin. In the culture that was allowed a 30-min recovery period, the rate of killing approached that of the untreated control culture (Fig. 3). This period is consistent with the time required to degrade the unstable proteins labeled during puromycin treatment.

Effects of induction of specific abnormal membrane proteins on rate of streptomycin killing. If puromycin peptides in the membrane fraction are responsible for more rapid streptomycin killing, the induction of other abnormal proteins may also increase streptomycin killing. We examined strain MW400, an \(rpsL^+\) derivative of strain MM18, which carries the genetic fusion \(malE-lacZ\) (a fusion of the N terminus of the periplasmic maltose-binding protein with the C terminus of the \(\beta\)-galactosidase protein [1]). This fusion protein is induced upon exposure to maltose. Upon induction, the synthesis and attempted export of the hybrid protein is initiated but not completed (1). The protein resides in the cytoplasmic membrane and interferes with the export of certain normal envelope proteins (18). Strain MW400 showed a rate of killing by streptomycin (100 \(\mu g/ml\)) similar to that of its parent strain, KD100 (Fig. 4A and B). The induction of the maltose regulon in KD100 did not affect its susceptibility to streptomycin (Fig. 4B). However, in MW400, when high levels of the fusion protein were induced by the addition of maltose, the rate of killing was enhanced (Fig. 4A) compared with that in KD100 or uninduced cultures of MW400.

A second set of studies examined *E. coli* strains that produced **malF-phoA** fusion proteins (2) under the control of the **tac** promoter. The strains synthesize the fusion proteins upon induction with isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) and insert the fusion proteins into the cytoplasmic membrane, with the alkaline phosphatase moiety correctly situated on the periplasmic face of the membrane (2). In contrast to MW400, in which induction of the **malE-lacZ** fusion protein eventually has serious effects on growth of the cell, the **malF-phoA** fusion strains show normal protein secretion after induction. In our studies we examined the rate of killing by streptomycin (50 \(\mu g/ml\)) of **malF-phoA** fusion strain MW512 in the presence and absence of IPTG and compared the data with those found with strain MW611, which contains a plasmid coding for the wild-type **malF** protein under control of the **tac** promoter (Fig. 5). The rates of killing by streptomycin in the two strains were identical and were not changed by the induction of the plasmid-encoded protein by IPTG. Similar rates of killing were found for another strain, MW520, carrying a different **malF-phoA** gene fusion (data not shown). These strains were more susceptible to streptomycin than were KD100 and its derivatives, however. Part of the increased susceptibility was the result of determinants on the pBR322-derived plasmids in strains MW611, MW512, and MW520, since strain MW601, which lacks this plasmid, was more resistant to streptomycin. Strain MW601 was still more susceptible than KD100, suggesting that other determinants in this genetic background may also influence its streptomycin susceptibility.
FIG. 4. (A) Effect of induction of the malE-lacZ fusion protein on the rate of killing of strain MW400 by streptomycin. An early-exponential-phase culture of MW400 was divided in half, and one portion was induced with 0.4% maltose for 2 h. Both cultures were centrifuged and suspended in BS medium containing streptomycin (100 μg/ml). □, Control culture; ▲, maltose-treated culture. These datum points are the averages of two to five separate experiments. (B) Effect of induction of wild-type maltose regulon on the rate of killing of strain KD100 by streptomycin. An early-exponential-phase culture of strain KD100 was divided in half, and one portion was induced with 0.4% maltose for 2 h. Both cultures were centrifuged and suspended in BS medium containing streptomycin (100 μg/ml). These datum points are the averages of two to eight separate experiments. ○, Control culture; ●, maltose-treated culture.

DISCUSSION

We showed that pretreatment of E. coli with a low concentration of puromycin (50 μg/ml) increased the rate of killing by streptomycin (Fig. 1). These data are consistent with previous reports that simultaneous treatment with puromycin and streptomycin leads to more rapid rates of killing (16, 27, 28). The effect of puromycin pretreatment is transient. Approximately normal rates of killing by streptomycin were restored after a 30-min incubation in antibiotic-free medium (Fig. 3). This time period is similar to the time required to degrade an unstable class of polypeptides (presumably, the puromycyl peptides) in these cells (Fig. 2). These findings support the suggestion (6–8) that low concentrations of puromycin can cause the formation of truncated puromycyl peptides that are sufficiently long to associate with the membrane and that these contribute to membrane leakiness and the potential for the rapid uptake of streptomycin. Although our studies did not directly show that puromycyl peptides reside in the membrane fraction, this model is consistent with a large body of evidence demonstra-
response (17). We showed that the induction of this fusion protein causes the cell to be killed by streptomycin at an increased rate (Fig. 4). Such findings are consistent with the model that abnormal membrane proteins cause increased leakiness of the membrane to small molecules such as streptomycin. An alternative explanation is that the induction of the fusion protein changes the membrane potential, although no direct evidence has been reported regarding such a change. In contrast to the results with the malE-lacZ fusion strain, the high-level production of malF-phoA fusion proteins did not alter the killing rate by streptomycin in strains MW512 and MW520 (Fig. 5). The transmembrane segment of malF in the amino terminus of the fusion protein contained in each of these strains localizes correctly and allows the alkaline phosphatase (phoA) domain to be correctly positioned within the periplasm (2). The induction of these fusion proteins with IPTG does not disrupt the processing of other membrane or periplasmic proteins and does not change the streptomycin susceptibility.

The rate of killing by streptomycin and other aminoglycosides can be influenced by the presence and/or activity of certain membrane proteins. These include the malE-lacZ protein, which has profound effects on the protein secretion apparatus; certain mutant alleles of the ATP synthase subunits, which cause changes in membrane permeability to protons (4, 15); and the plasmid-encoded tetracycline resistance protein, which alters membrane permeability to cations (12, 19). These data are consistent with the model (6-8) that abnormal membrane proteins which cause alterations in membrane permeability (and, possibly, membrane potential) play an essential role in the bactericidal action of aminoglycosides.

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LITERATURE CITED