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INHIBITION OF INITIATION OF DNA SYNTHESIS BY AMINOGLYCOSIDE ANTIBIOTICS

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In a dnaC^{ts} mutant of E. coli, the reinitiation of DNA synthesis, which occurred by the shift of the culture from a restrictive temperature to a permissive temperature, was markedly prevented by habakacin, dibekacin, kanamycin, and gentamicin. On the contrary, chloramphenicol did not inhibit the reinitiation synthesis for 30 min. In a parallel experiment, leucine uptake into protein was profoundly blocked by chloramphenicol, but only slightly by habekacin. Habekacin did not significantly affect DNA elongation of the cells at a restrictive temperature. We propose that inhibition of initiation of replication by aminoglycoside antibiotics is related to their lethality.

Aminoglycoside antibiotics show pleiotropic effects on bacteria. They induce a series of molecular events, including inhibition of ribosomal functions and membrane damage. Although the ribosome is the main target, the specific reason for the bactericidal nature remains to be determined (for reviews, see references 1-5). We report here that aminoglycosides prevent initiation of DNA synthesis without affecting DNA elongation.

Of the antibiotics used, habekacin, or 1-[(S)-4-amino-2-hydroxybutyryl]dibekacin, is a novel drug with a broad antibacterial spectrum, and exhibits a mode of action and bacterial uptake similar to that of other deoxystreptamine-containing aminoglycosides (6,7).

MATERIALS AND METHODS

Chemicals. Habekacin, dibekacin, and kanamycin were supplied by Meiji Seika Kaisha, Ltd., Tokyo, and chloramphenicol by Sankyo Co., Ltd., Tokyo. Gentamicin was a product of Schering Corporation, Bloomfield, New Jersey. [^{3}H]Thymidine (25 Ci/mmol) and [^{3}H]leucine (138 Ci/mmol) were purchased from Amersham Japan, Tokyo.
Bacterial strain. A temperature-sensitive replication initiation mutant of E. coli K-12, PC2 (dnaC^{ts}, leu-6, thyA47, dra-3, str-153) (9) was generously provided by Dr. K. Nagai, Department of Agricultural Chemistry, University of Tokyo, Tokyo.

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Medium. The medium used contained per liter: 2 g of glucose, 4 g of casamino acids, 40 mg of thymine, 0.54 g of NaCl, 0.3 g of KCl, 1.1 g of NH_4Cl, 15 mg of $CaCl_2 2H_2O$, 203 mg of $MgCl_2 6H_2O$, 0.2 mg of $FeCl_3 6H_2O$, 87 mg of KH_2PO_4, 22.7 mg od Na_2SO_4, and 12.1 g of Tris-HCl (pH 7.5).

DNA synthesis. The rate of DNA synthesis was measured as the rate of incorporation of [^{3}H]thymidine into cold TCA-precipitable counts after a 1 - 2 min pulse. The bacterial culture of 0.5 ml was transferred to each tube, containing 0.5 - 1 μCi of [^{3}H]thymidine. Pulses were terminated by addition of cold 5% TCA. The TCA-insoluble radioactivity, collected on glass filter and washed with 10 ml of 5% TCA, was counted in a toluene-based scintillation fluid, using a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Inhibition by aminoglycosides of reinitiation of DNA synthesis. When an exponentially growing culture of a *dnaC*^{ts} mutant (PC2) was shifted from a permissive temperature (30°C) to a restrictive temperature (40°C), DNA elongation terminated in 120 min. Whe the cells were subsequently shifted back to 30°C, reinitiation of DNA synthesis occurred after a short lag. Habekacin markedly blocked the reinitiation synthesis, when added to the culture 5 min prior to the return to 30°C. Approximately 60 - 70% inhibition was observed in 5 - 20 min (Fig. 1). On the contrary, chloramphenicol did not significantly affect the reinitiation for 30 min, suggesting that *dnaC* protein, inactivated by the shift to the restrictive temperature, renatured and reinitiated DNA synthesis upon return to the permissive temperature. The antibiotic concentrations used completely inhibited bacterial growth (data not shown).

In a simultaneous experiment, [^{3}H]leucine incorporation into protei fraction was markedly prevented by chloramphenicol, but only slightly by habekacin (Fig. 2). The codon misreading activity seemed to influence the effect of habekacin. The results suggest that the inhibition of reinitiation of replication does not merely result from a general blockage of protein synthesis, but also from other effect.

The inhibition of reinitiation of DNA synthesis was also found with other aminoglycoside antibiotics: dibekacin, kanamycin, and gentamicin (Table 1). More marked inhibition was demonstrated with

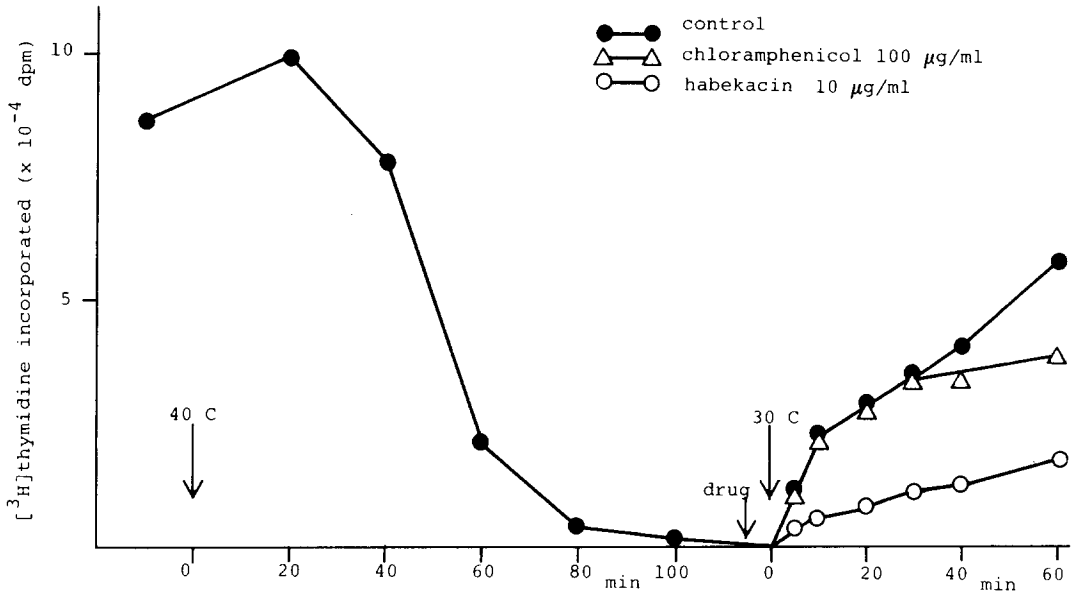

Fig. 1. Effects of habekacin and chloramphenicol on reinitiation of DNA synthesis in a dnaC^{ts} mutant. E. coli PC2 was grown at 30 C, and shifted to 40 C at a logarithmic phase of growth (4 x 10^8 cells/ml). After 120 min the culture was shifted back to 30 C. The antibiotic was added to the cells 5 min prior to the return to 30 C. The rate of DNA synthesis was measured as the rate of uptake of [^{3}H]thymidine (1 μCi/0.5 ml) after a 1 min pulse.

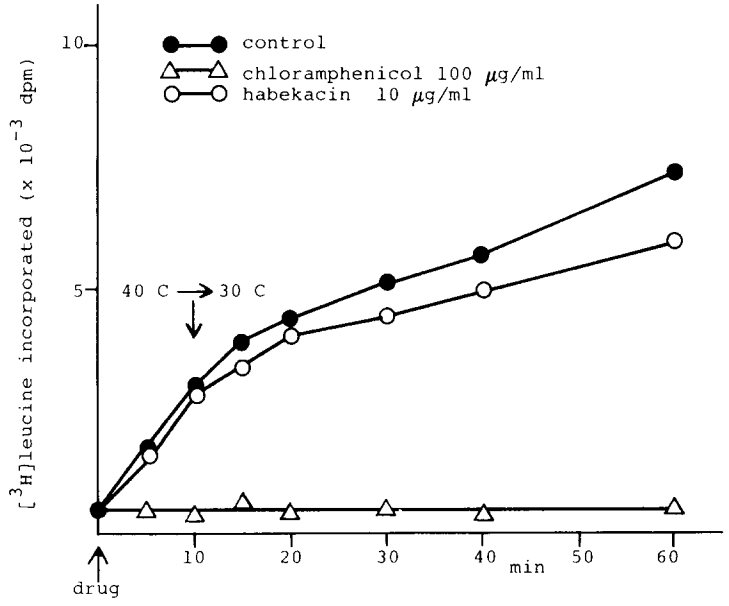

Fig. 2. Effects of habekacin and chloramphenicol on leucine uptake into protein in a dnaC^{ts} mutant. E. coli PC2 was cultured at temperatures described in the legend of Fig. 1. The drug and [^{3}H]leucine (10 μCi/ml) were added to the cells 10 min prior to the return to 30 C. The hot TCA-insoluble radioactivity of 70 μl of the culture was determined in each experiment.

Table 1. Effects of aminoglycoside antibiotics on reinitiation of DNA synthesis in a *dnaC*^{ts} mutant

Antibiotic		% Inhibition
Habekacin	10 μg/ml	94
Dibekacin	10	86
Kanamycin	100	97
Gentamicin	10	96

The procedure followed the one described in the legend of Fig. 1, except that pulse labeling was performed for 2 min with 0.5 μCi/0.5 ml of [^{3}H]thymidine. The antibiotics were added to the cells 10 min prior to the return to 30 C. DNA synthesis was determined 15 min after the shift to 30 C.

habekacin than that in the previous experiment, because the drug was added to the culture 10 min prior to the return of the cells to the permissive temperature (30°C).

Effect of habekacin on elongation of DNA synthesis. Effects of habekacin and chloramphenicol on DNA synthesis of a *dnaC*^{ts} mutant were studied at a restrictive temperature (40°C). When the exponentially growing culture was shifted from 30° to 40°C, the initiation synthesis ceased in a short period. After 10 min the drug was added to the culture, to study the effect on DNA elongation. Habekacin, as well as chloramphenicol, did not significantly affect DNA synthesis (Fig. 3). The results suggest that neither antibiotic significantly affects DNA elongation.

DISCUSSION

The *dnaC* gene is thought to code for protein required for the initiation of chromosomal DNA replication in *E. coli*. Current studies with a *dnaC*^{ts} mutant present evidence that aminoglycoside antibiotics prevent initiation of DNA synthesis. Several mechanisms might result in the inhibition of initiation. Firstly, the antibiotics may interfere with synthesis of a protein factor required prior to initiation of replication. This is not consistent with the finding that chloramphenicol, another ribosomal inhibitor, did not significantly affect initiation of DNA synthesis in a parallel

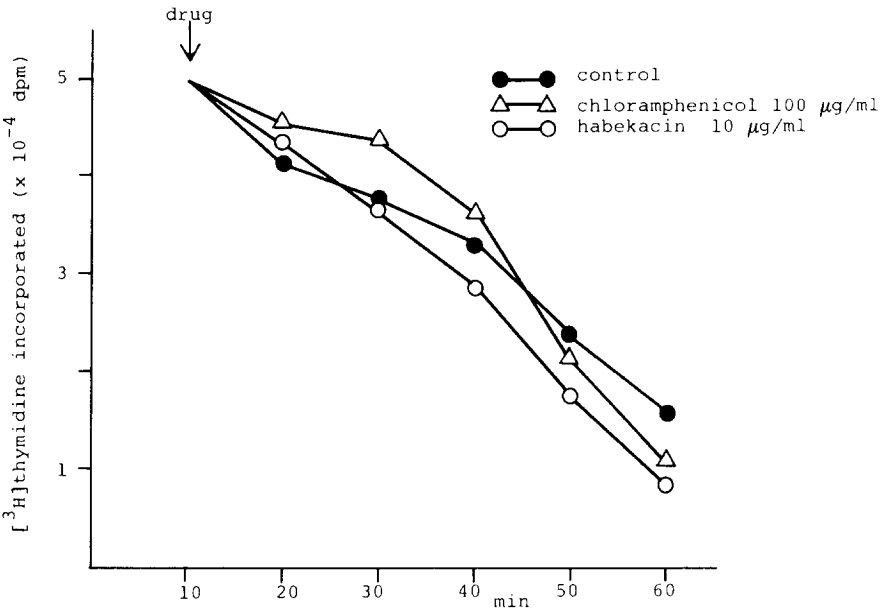

Fig. 3. Effects of habekacin and chloramphenicol on DNA elongation in a *dnaC*^{ts} mutant. *E. coli* PC2 was grown at 30 C, and shifted to 40 C at an exponential phase of growth (3 x 10^8 cells/ml). After 10 min the drug was added to the culture. The rate of DNA synthesis was determined by pulse labeling with [^{3}H]thymidine (0.5 μCi/0.5 ml) for one min.

experiment. Hanna and Carl (9) reported that the reinitiation of DNA synthesis did not require either protein or RNA synthesis. Secondly, binding of aminoglycosides to DNA might cause inhibition of initiation. This explanation is also unlikely, because the antibiotics do not block DNA elongation. Finally, aminoglycosides may act by disrupting the DNA-membrane attachment site, which participates in initiation of replication (8). This hypothesis seems to be in accord with the results so far obtained.

The molecular mechanism, involved in aminoglycoside action, especially the reason for their lethal effects, remains to be determined, in view of the pleiotropic effects on bacteria, including interference with protein synthesis and membrane damage. It is widely accepted that inhibition of ribosomal functions is the most important mechanism of action of aminoglycosides. However, a general inhibition of protein synthesis and an ability to stimulate codon misread-

ing are not the specific reason for the lethal nature of aminoglycosides (1-5). Chloramphenicol, tetracyclines, macrolides, and other protein synthesis inhibitors show primarily bacteriostatic effects. Here we propose the hypothesis that the prevention of initiation of DNA synthesis, possibly in cooperation with the disturbance of protein synthesis, may result in cell death. Hancock (5) assumed that the disruption by streptomycin of the DNA-membrane attachment site may be related to the EDP-II (energy-dependent phase II uptake) and lethality. In this connection, it is worthy of note that nalidixic acid, an inhibitor of DNA synthesis, shows only bactericidal activity (for reviews, see references 10 and 11).

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