INHIBITION OF DNA REPLICATION AND MEMBRANE TRANSPORT OF SOME NUTRIENTS BY CLAZAMYCIN IN *ESCHERICHIA COLI*

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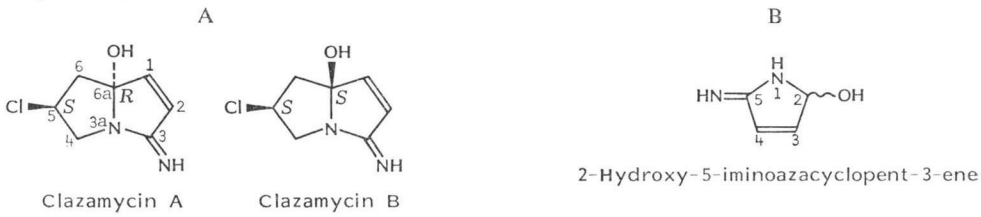
(Received for publication August 31, 1983)

Growth of *Escherichia coli* in a nutrient medium was inhibited by 100 μg/ml of clazamycin and at this concentration, the viable cell number decreases slowly. Elongated cells were observed in the treated cultures. The bactericidal activity was abolished by high concentrations of either sucrose or sorbitol but not by chloramphenicol. Non-growing cells suspended in a medium devoid of both carbon and nitrogen sources were killed by clazamycin more rapidly than cells in a rich medium. Incorporation of radioactive thymidine, uridine, leucine and *N*-acetylglucosamine into cellular macromolecules was inhibited to a similar extent. Permeability of *N*-acetylglucosamine and leucine was blocked by clazamycin. On the other hand, membrane transport of thymidine was only slightly inhibited. Thymidine-derived radioactivity accumulated as dTTP in the cells suggesting that DNA synthesis was blocked at the polymerization step. DNA synthesis in toluene-treated cells was also sensitive to clazamycin while the repair DNA synthesis induced by bleomycin in these cells was not. DNA-repair deficient mutants of *E. coli* were as sensitive to clazamycin as their DNA-repair proficient counterparts.

Clazamycin was isolated from culture filtrates of *Streptomyces* No. MF990-BF4 and its structure was determined (Fig. 1A). The antibiotic is active against some Gram-positive and Gram-negative bacteria and prolongs the life span of mice inoculated with cells of L-1210, a mouse leukemia.[1] Clazamycin shares a common structure with 2-hydroxy-5-iminoazacyclopent-3-ene (HIACP, Fig. 1B), which has a similar antibacterial spectrum with somewhat lower activities. HIACP was reported to cause elongation of bacterial cells implying some interference with the cell surface.[2] Considering the similarity in structures and biological activities, we investigated clazamycin activity on the cell surface of bacteria. Our studies indicate that clazamycin, possibly by acting on a component(s) of the cytoplasmic membrane, causes (1) rapid inhibition of membrane transport of specific nutrients, such as *N*-acetylglucos-

Fig. 1. Structures of clazamycins and HIACP.

Clazamycins A and B equilibrate at a molar ratio of 3/2 in neutral aqueous solution. All the experiments were conducted with this mixture solution.



amine and leucine, (2) rapid inhibition of polymerization process of DNA replication, and (3) slowly progressing disintegration of cell walls. HIACP also inhibited DNA replication. As an extension, we found that the cytotoxicity of clazamycin to mouse leukemia cells could also be ascribed to an effect on the cell membrane.

Materials and Methods

Bacterial Strains

Unless otherwise indicated, *Escherichia coli* K12 was used as the test strain. Mutants of K12 used were BEM11 (*tolC*, F^-, *thr, leu, pro, his, thi, arg, lac, gal, ara, xyl, mtl, tsx, str, sup*), BE1121 (*recA1*, *his*$^+$, otherwise the same as BEM11) and BE1186 (*uvrA, ruv*-9, *his*$^+$, otherwise the same as BEM11), kindly supplied by Dr. N. OTSUJI, Univ. of Kyushu, and JE6269 (*polA1*, *purE, trp, lys, proC, leu, thi, lacZ, xyl, ara, tonA, tsx, str, sup*) by Dr. Y. HIROTA, National Institute of Genetics, Shizuoka.

Culture Media

Medium A consisted of 1% Polypeptone (Daigo), 0.5% Bacto yeast extract (Difco), 0.5% NaCl, 0.5% glucose and water to volume, pH adjusted to 7.2. Medium B consisted of 0.5% meat extract (Kyokuto), 1% Polypeptone (Daigo), 0.5% NaCl and water to volume, pH adjusted to 7.2. Medium C consisted of 1% Casamino acids, 0.3% NaCl, 0.2% glycerol, 0.025% $MgSO_4 \cdot 7H_2O$, 0.0011% $CaCl_2$, 0.2% KH_2PO_4, 0.73% Tris-base and water to volume, pH adjusted to 7.4.

Incorporation of Radioactive Precursors into Acid-insoluble Materials of Cells

Cells were grown at 37°C with shaking in medium C to 0.1 $A_{600\,nm}$ (cell suspension). An assay mixture of 1.0 ml was made up of 0.8 ml of the cell suspension and appropriate volumes of aqueous solutions of the following components to make their final concentrations; 0.1 mM deoxyadenosine, 0.4 M sucrose, 50 μg/ml of clazamycin (water in control), and either 0.5 μCi/ml [^{3}H]thymidine, 0.25 μCi/ml [^{3}H]-uridine, 1.25 μCi/ml [^{3}H]leucine, or 0.25 μCi/ml *N*-[^{14}C]acetylglucosamine. Cells were exposed to clazamycin 2 minutes before initiation of labeling at 37°C. At the times indicated, 100 μl samples were withdrawn, placed onto Whatman 3 MM filter discs (2.5 cm in diameter) and submitted to determination of acid-insoluble radioactivity.

Membrane Transport and Intracellular Conversion of Radioactive Precursors

Cells were grown in medium C to 0.2 $A_{600\,nm}$, collected by centrifugation and suspended in fresh medium C at 10^9 cells/ml. Two to five ml of the cell suspension (depending on the size of experiment) received clazamycin at the final concentration of 100 μg/ml and the mixture was incubated at 37°C for 10 minutes with shaking. Either [^{3}H]thymidine (2 μCi/ml of the mixture) or *N*-[^{14}C]acetylglucosamine (0.5 μCi/ml of the mixture) was then added and incubation was continued. At 20, 40 and 80 seconds of incubation, 0.5 ml samples were withdrawn, each was placed on a 0.2 ml layer of 84:16 (v/v) mixture* of silicon oil and liquid paraffin in a microcentrifuge tube, and spun at 15,000 *g* for 1 minute in an Eppendorf microcentrifuge. The uppermost layer was discarded, the surface of the tube and the oil layer was gently washed with water, and the oil layer was removed by aspiration leaving the cell pellet, which was dissolved in 0.2 ml of 0.5 N KOH and submitted to the determination of radioactivity ('membrane transport', circles in Figs. 5 and 6). At 80 and 300 seconds of incubation, 0.5 ml samples were withdrawn, each was mixed with 5 ml of 5% TCA and processed for the determination of radioactivity incorporated into acid-insoluble materials of cells (triangles in Figs. 5 and 6).

For analysis of [^{3}H]thymidine-derived radioactivity in the acid-soluble pool of cells, a 0.5 ml sample was withdrawn at 80 seconds of incubation, placed on a double-layer consisting of 160 μl of the oil mixture (see above*) and 100 μl of 10% PCA solution, and spun at 15,000 *g* for 1 minute. After removing the top layer (derived from the sample) and the oil layer by aspiration, 60 μl of the PCA-soluble fraction was withdrawn** and mixed with a small volume of carrier solution dissolving trace amounts of unlabeled thymidine, dTMP, dTDP and dTTP and with 240 μl of tricaprylyl tertiary amine (Alamine; Henkel Co., Minn., U.S.A.). After centrifugation at 15,000 *g* for 1 minute, a 30 μl sample was applied in a streak to a PEI cellulose thin layer which was developed with (1) 0.5 M HCOONa for 8 minutes (2.5 cm from the origin), (2) 2.0 M HCOONa for 30 minutes (7.0 cm from the origin) and (3) 4.0 M HCOONa

for 110 minutes (15 cm from the origin).[3] Thymidine (Rf 0.84), dTMP (Rf 0.71), dTDP (Rf 0.32) and dTTP (Rf 0.12) were localized under UV light and the corresponding areas were scraped, extracted with 0.5 N KOH and radioactivities were determined. After removal of PCA soluble fraction (see preceding page**), each PCA-insoluble pellet was washed with 5% PCA solution, dissolved in 500 μl of 0.5 N KOH and radioactivity was determined.

DNA Synthesis in Toluene-treated *E. coli*

Experiments on replicative DNA synthesis were performed as reported[4] with minor modifications. In brief, growth of JE6269 was terminated at 0.4 $A_{600\,nm}$ by rapid chilling, cells were collected by centrifugation, treated with 1% toluene, suspended in a buffer and used for determination of DNA synthesis. An assay mixture contained, in 100 μl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 25 μM each of dNTP's except dTTP, 0.15 μCi of [^{3}H]dTTP (0.6 mCi/mmol), 150 mM KCl, 1.5 mM ATP, a test compound at a desired concentration, 6×10^8 toluene-treated cells (last added), and water to volume. After incubation at 30°C for 30 minutes or 60 minutes, 2 ml of cold 5% TCA-20 mM sodium pyrophosphate was added to the mixture. Acid-insoluble materials of a mixture were collected on a GF/C filter disc, washed, dried and determined for radioactivity. Experiments on repair synthesis of DNA were performed as above except that BEM11 was the tester strain, bleomycin B6 was added at 20 μg/ml and ATP was removed from the reaction mixture.

Results and Discussion

Bactericidal Effects

When exponentially growing cells of *E. coli* were exposed to 100 μg/ml of clazamycin, cell growth ceased almost immediately (Fig. 2). Turbidity of the culture did not increase after addition of clazamycin. Most of the cells remained viable for 2 hours and then began to die slowly. If 0.25 M (or 0.4 M) sucrose was added to the culture medium, only a few cells were killed during the 4-hour incubation (Fig. 2). Sorbitol at the same concentration range showed a similar protective effect (data not shown). After expanded incubation, clazamycin appeared to cause cells to become fragile, ultimately leading to death in the absence of an appropriate osmotic stabilizer.

Fig. 2. Protection of *E. coli* by sucrose from the bactericidal effect of clazamycin.

E. coli K12 cultured in medium A 'with' (open symbols) or 'without' (solid symbols) 0.25 M sucrose. The cultures receiving clazamycin are shown by triangles. At the time indicated, samples were withdrawn from the cultures, appropriately diluted and submitted to colony counting by the pour plate method.

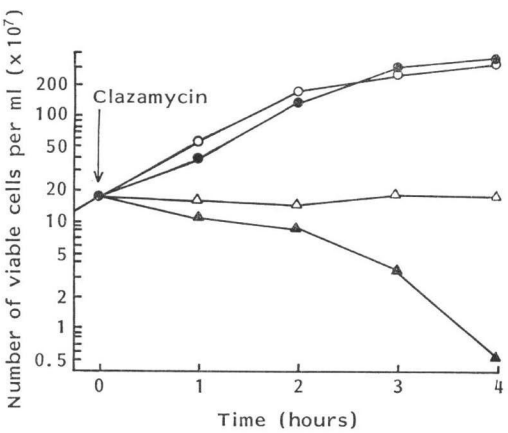
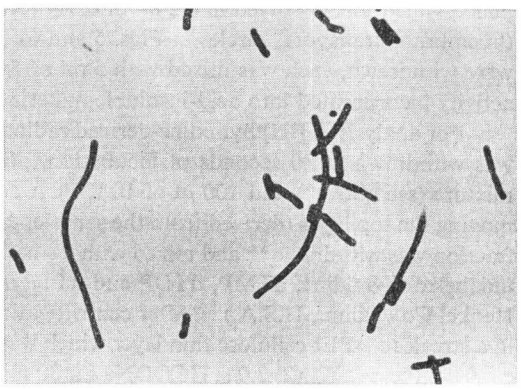


Fig. 3. Morphological change of *E. coli* K12 exposed to clazamycin.

Smears were prepared from 4-hour cultures, stained with crystal violet.



Formation of Filamentous Cells

Microscopic examination of a sample taken from a culture exposed to clazamycin (100 μg/ml) for 3 hours showed filamentous cells (Fig. 3). Elongation of bacterial cells is a characteristic of drugs which inhibit the synthesis of either DNA or some component of the cell surface. DNA inhibitors which cause cell elongation are exemplified by mitomycin,[5] bleomycin,[6] nalidixic acid,[7] *etc.*, while cell surface inhibitors are exemplified by penicillins,[8] vancomycin,[9] bicyclomycin,[10] *etc.* The molecular mechanism of each drug within a group, DNA or cell surface inhibitors, are significantly different. Clazamycin caused only negligible lethality by short treatment, whereas cell growth was totally inhibited, suggesting that the drug was readily released from the target site by the dilution performed before colony counting procedures. If the DNA template is the target for clazamycin, DNA-repair deficient mutants will be more sensitive than DNA-repair proficient strains.[11] The minimum inhibitory concentrations of clazamycin were in the range of 12.5 to 25 μg/ml with DNA-repair deficient mutants, BE1121 and BE1186, and DNA-repair proficient counterpart. Furthermore, the DNA polymerase reaction with isolated enzyme was not inhibited by clazamycin (see below). The drug-induced cell fragility and lack of activity against DNA replication implicate a cell surface component as a target site.

Higher Susceptibility of Non-Growing Cells

In contrast to penicillin and other cell wall inhibitors, clazamycin (100 μg/ml) killed non-growing cells more rapidly than growing cells. The data with either the addition of chloramphenicol or the removal of essential nutrients are shown in Table 1. The action of clazamycin on non-growing cells suggests the disruption of a structural component ultimately leading to cell disintegration.

Effect on Incorporation of Radioactive Precursors into Cellular Macromolecules

Macromolecular synthesis was measured by adding radioactive thymidine, uridine, leucine or *N*-acetylglucosamine to exponentially growing cells of *E. coli* in the presence (100 μg/ml) or absence of clazamycin. The incorporation of radioactivity into acid-insoluble materials of the cells was followed with time. Clazamycin inhibited the incorporations of all the precursors to a similar extent (Fig. 4). At lower concentrations of clazamycin, the inhibition of all precursors was reduced to a similar degree. The apparently uniform inhibition of incorporations was probably not due to a nonspecific damage on the cell membrane since most of the cells were still alive after exposure (Fig. 2). Incorporation of these

Table 1. Effect of clazamycin on viability of *E. coli* under non-growing conditions.

Condition	Incubation time*			
	1 hour	2 hours	3 hours	4 hours
Non-growing				
C and N sources omitted from medium C	nd	0.015	0.00021	0.0001> (1.5)
Chloramphenicol added to medium C	1.1	0.015	nd	0.01> (0.7)
Chilled (4°C) in medium B	1.3	nd	0.91	0.82 (0.96)
cf.				
Growing				
Medium B	0.73	0.51	nd	0.034

* Each figure represents a surviving fraction determined by colony counting (see the legend to Fig. 2). The parenthesized figures at 4 hours represent "without clazamycin".
nd: Not determined.

Fig. 4. Effect of clazamycin on cellular macromolecule synthesis in *E. coli*.
Open and solid circles represent 'with' and 'without' clazamycin, respectively. For details, see Methods.

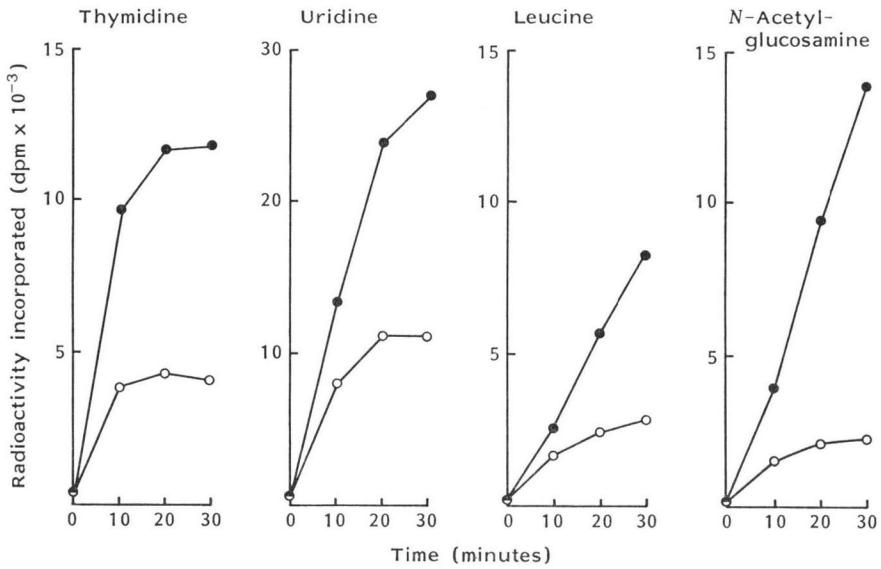


Fig. 5. Effect of clazamycin on *N*-[^{14}C]acetylglucosamine uptake by cells of *E. coli* and on the successive incorporation into macromolecules.
Open and solid symbols represent 'with' and 'without' clazamycin, respectively. Circles represent radioactivity in whole cells (membrane transport) while triangles represent that in acid-insoluble materials of cells. For details, see Methods.

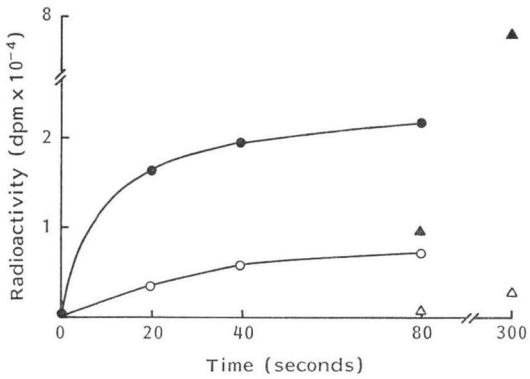
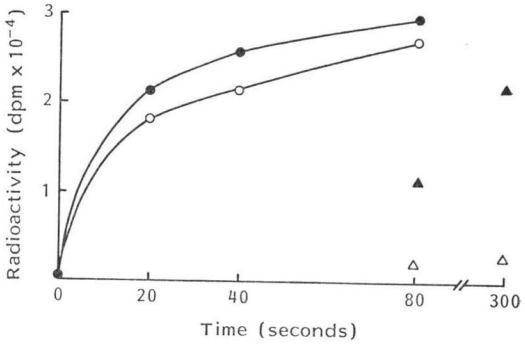


Fig. 6. Effect of clazamycin on [^{3}H]thymidine uptake by cells of *E. coli* and on the successive incorporation into DNA.
See legend of Fig. 5.



precursors into cellular macromolecules could be divided into 3 steps; (1) transport across the cell membrane, (2) conversion to the ultimate precursor of each macromolecule and (3) polymerization. Additional experiments were conducted to define which of these steps was inhibited.

Inhibition of Membrane Transport of *N*-Acetylglucosamine and Leucine

Clazamycin inhibited membrane transport of *N*-acetylglucosamine (Fig. 5). The blockade at this process seems to be primarily, if not solely, responsible for the inhibition of incorporation of this pre-

Table 2. Effect of clazamycin on metabolic conversion of [^{14}C]thymidine in *E. coli*.

Condition	Acid-soluble (dpm)					Acid-insoluble (dpm)
	Thymidine	dTMP	dTDP	dTTP	Total	
Control	743	1,050	1,118	1,618	4,529	3,101
+ Clazamycin	1,746	914	1,506	5,546	9,712	659

cursor. Membrane transport of leucine was also inhibited in similar manner. These results, together with the protective effect of sucrose, indicate that there is an interaction between clazamycin and the cell membrane. The membrane transport systems for some sugars and amino acids are localized at least in part in the cytoplasmic membrane.[12] Therefore, clazamycin probably acts on a component of the membrane resulting in inhibition of specific transport systems.

Inhibition of Polymerization Step of DNA Synthesis

In contrast to the membrane transport of *N*-acetylglucosamine and leucine, the transport of thymidine was refractory to clazamycin (Fig. 6). The radioactive thymidine taken into the cells, however, was not efficiently incorporated into DNA in the presence of clazamycin. The blockade was further defined by analyzing the radioactivity in the acid-soluble pool. Thymidine-derived radioactivity accumulated as dTTP in the acid-soluble pool in the presence of clazamycin (100 μg/ml), indicating that the antibiotic inhibited the polymerization step (Table 2). Toluene treatment of bacteria is one of the most faithful systems *in vitro* for studying DNA replication, especially the polymerization process, *in situ*.[4] As Table 3 shows, replicative DNA synthesis in toluene-treated cells was as sensitive to clazamycin as that in toluene-untreated cells confirming that the target of clazamycin was the polymerization step. These results also indicated that the target site survived the toluene-treatment. The repair synthesis of DNA induced by bleomycin in toluene-treated cells was not inhibited by clazamycin. In addition, DNA synthesis catalyzed by purified enzyme from *Micrococcus luteus* (E. C. 2.7.7.7) with calf thymus DNA as a template was not inhibited by clazamycin.

Table 3. Inhibition by clazamycin and HIACP of DNA replication in toluene-treated *E. coli*.

Test compounds (μg/ml)		Reaction time	
		30 minutes	60 minutes
None (control)		2,972 dpm	5,376 dpm
Clazamycin	20	963	4,019
	100	723	1,728
	500	−140	−33
HIACP	200	2,339	3,849
	500	1,397	2,571
Novobiocin	5	−437	−244

Radioactivity at '0 minute' of the control run has been subtracted from each measurement.

Since uridine and thymidine are known to share a common membrane-transport mechanism,[13] clazamycin inhibition of [^{14}C]uridine incorporation also probably involves the polymerization step. Clazamycin at 100 μg/ml had no effect on RNA synthesis *in vitro* catalyzed by RNA polymerase isolated from *E. coli* MRE600. All the results presented so far could be interpreted as indicating that the target of clazamycin is a component of the cytoplasmic membrane which is closely associated with integrity of the cell walls, with the membrane transport system(s) of some nutrients, and with the replication machinery of DNA.[14] Repair synthesis of DNA must be independent of the target molecule. HIACP, the structurally related antibiotic (Fig. 1B), also inhibited DNA replication in toluene-treated cells (Table 3) suggesting that the common structure between the two antibiotics is important for all these effects. Clazamycin and HIACP would provide a clue for elucidation of a membrane component involved in DNA replication in *E. coli*.

Clazamycin may also act on mammalian cells by interfering with membrane functions. Cells of L-1210, a mouse leukemia, became permeable to trypan blue after an hour's exposure to 4 to 20 μg/ml clazamycin (referred to as 'concentration for membrane damage') at 37°C. This concentration range was only about 5 times higher than that required for 50% growth-inhibition on 2-days exposure of *in vitro* cultured cells (1 to 2 μg/ml, referred to as 'concentration for cytotoxicity'). The index of 'concentration for membrane damage'/'concentration for cytotoxicity' was generally much higher for antitumor antibiotics having other mechanisms of action (for instance, adriamycin, bleomycin and 1,3-bis(2-chloroethyl)-1-nitrosourea).[15] Details of the studies with mammalian cells will be published separately.

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