Bactericidal Effect of 5-Azacytidine on *Escherichia coli* Carrying *Eco*RII Restriction-Modification Enzymes

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5-Azacytidine was found to be bactericidal to *Escherichia coli* carrying plasmids specifying *Eco*RII restriction-modification systems, but not to the same strains lacking these plasmids. Of other base analogs tested, only 5(β-D-ribofuranosyl)isocytidine had similar, although weaker, effects. Plasmids that had lost the *Eco*RII restriction-modification system did not confer sensitivity to 5-azacytidine. Mutants defective in the restriction function remained sensitive to the toxic effects of the drug; however, a mutant defective in the modification function lost most of the sensitivity to 5-azacytidine. For the bactericidal effect to be seen, the cells had to be growing; cells in the stationary phase of growth were not killed by the drug. The drug inhibited the methylase enzyme, and an inhibitor of the enzyme could be detected in vitro in extracts of cells that had been treated with 5-azacytidine. This inhibitor was destroyed by DNase, and antibiotics such as coumermycin and nalidixic acid inhibited its formation. Coumermycin but not nalidixic acid antagonized the bactericidal effect of the drug; however, coumermycin was more effective in preventing the inhibition of the methylase by 5-azacytidine than was nalidixic acid.

5-Azacytidine inhibits several bacterial DNA (cytosine-5)methylases, but not the DNA (N^6-adenine)methylase present in the same organism (9, 10). For inhibition to occur in vivo, the cells must be growing; the enzyme is not inhibited when resting cells are treated with the drug. The inhibition could not be produced by treating cell extracts with 5-azacytidine; however, DNA (cytosine-5)methylases could be inhibited irreversibly, in vitro, if they were incubated with 5-azacytosine-containing DNA. For inhibition to occur, the DNA sequence that would otherwise be methylated had to be intact because prior digestion of 5-azacytosine-containing DNA with the complementary restriction enzyme destroyed the inhibitory activity of the DNA for the methylase (10).

Modification of DNA protects that DNA from the complementary restriction enzyme (22). If inhibition of the modification methylase should eventually lead to the presence of completely unmodified DNA in the cell, such DNA would be degraded by the restriction endonuclease. Thus, cells containing cytosine-dependent restriction-modification systems should not form viable progeny when grown in the presence of 5-azacytidine.

5-Azacytidine is also an inhibitor of bacterial cell growth. This inhibition is believed to be due to incorporation of the drug and its deaminated product, 5-azauridine, into mRNA, with resultant inhibition of protein synthesis; however, DNA synthesis is only slightly affected (7, 21).

The inhibition of growth caused by the drug can be readily reversed by removing it from the growth medium (4).

I have now tested cells containing the *Eco*RII restriction-modification system for the ability to resume growth after being treated with 5-azacytidine for a brief period of time. These cells lose viability after removal of the drug. However, the bactericidal effect is due not to the presence of the restriction enzyme, but is a function of the methylase.

MATERIALS AND METHODS

5-Azacytidine, 5-fluorouracil, 5-azauracil, 5-azacytosine, and 6-azauridine were obtained from Sigma Chemical Co., St. Louis, Mo. 5-Fluorocytidine was a gift of W. E. Scott, of Hoffman-LaRoche, Inc., Nutley, N.J. 5(β-D-ribofuranosyl)isocytosine (ψ-isocytidine) was a gift of K. A. Watanabe, Sloan-Kettering Institute for Cancer Research, Rye, N.Y. 5-Fluorouridine and 6-azacytidine were obtained from Calbiochem, La Jolla, Calif. Coumermycin was obtained from Godfrey Science and Design, White Plains, N.Y.

Bacterial strains, plasmids, and growth conditions. Table 1 lists the bacterial strains and plasmids used. The plasmids N3, R15, R390, and R459 specify the *Eco*RII restriction-modification [Res⁺ Mod⁺ (RII)] system. The modification enzyme methylates the second cytosine in the sequence CC$^{\mathrm{T}}_{\mathrm{A}}$GG protecting the DNA from hydrolysis by the restriction endonuclease (22). *Escherichia coli* strain K-12 and its derivatives contain an enzyme specified by the *mec* gene product that methylates the identical cytosine (12, 13).

TABLE 1. Bacterial strains and plasmids[a]

E. coli or plasmid strain	Relevant properties	Source
E. coli		
B	Wild-type Luria strain	ATCC 11303
K-12 W6	met relA mec[+]	ATCC 25019
K38	mec[+]	N. Zinder (17)
F1100	Su[+] endI thi mec[+]/ F[+]	S. Hatt-man (13)
F1100 mec		S. Hatt-man (13)
SΦ441	cdd-5 upp-11 relA1 metB1 rpsL254	B. Bach-mann (11)
J53	pro met	N. Datta (6)
Plasmid		
pBR322	Ap Tc	D. Buchha-gen (3)
R15	Su Sm Res[+] Mod[+] (RII)	R. J. Rob-erts (24)
R390	Tc Cm Su Sm Ap Res[+] Mod[+] (RII)	N. Datta (6)
R459-S1[b]	Ap Sm Sp Tc Cm Su Km	N. Datta (14)
N3	Tc Su Sm Res[+] Mod[+] (RII)	S. Hatt-man (24)
N3 Res[-] Mod[+] (RII)		S. Hatt-man (23)
N3 Res[-] Mod(Am) (RII)		S. Hattman (23)
R459-S2	Su Sm	This paper
R390-S1	Cm Su Sm Ap Res[+] Mod[+] (RII)	This paper
R390-S2	Su Ap	This paper

[a] Plasmids were transferred between strains as described by Bannister and Glover (2). Deletion mutants of plasmid R390 in strain J53 were selected by two cycles of growth for 30 min each in 5-azacytidine (20 μg/ml), followed by washing the cells and growing them overnight in fresh medium. Su, sulfonamide; Sm, streptomycin; Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Res[+] Mod[+], restriction-modification; Am, amber mutation. Other abbreviations used are those of Bachmann and Low (1) and Novick et al. (20).

[b] R459, received as Res[-] Mod[-] (RII).

Minimal A medium (19) was supplemented with 1% Casamino Acids. The medium was supplemented with thiamine (1 μg/ml) when required. Bacteria were plated on yeast extract tryptone medium containing 1.5% agar (19).

The bacteria were cultivated in 10 ml of medium in 250-ml side-arm flasks (Bellco) in a reciprocating shaker at 37°C. Growth was recorded by turbidity readings in a Klett-Summerson colorimeter with a no. 54 filter. When the cells reached 50 Klett units (10^8 cells/ml), equal portions were removed and placed in 16 × 100-mm tubes containing drug as indicated. The tubes were shaken at 37°C. Samples were removed at the indicated times and plated for viable cells.

Phage restriction and modification and efficiency of plating (EOP) were determined as described by Wood (25). Plasmid transfer was performed as described by Bannister and Glover (2), and transfection was performed as described by Cohen and Chang (5).

Enzyme preparation. Cells were grown in 25 ml of medium as described above, harvested by centrifugation, and washed once with buffer containing 0.01 M Tris-hydrochloride and 1 mM EDTA, pH 8. The cells were suspended in 1 ml of buffer containing 0.01 M potassium phosphate, pH 7, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM sodium azide, 0.4 M sodium chloride, and 25 μg of phenylmethylsulfonyl fluoride per ml and sonicated with two 30-s bursts in a Branson sonicator. The extract was centrifuged at 40,000 rpm in a Beckman 40 rotor for 60 min, and the supernatant was used as a source of enzyme or inhibitor.

Enzymes assays. DNA methylase activity measured the incorporation of radioactivity from S-adenosyl-[methyl-^{14}C]methionine into alkali-stable, acid-precipitable material resistant to deproteinization as described by Kalousek and Morris (15), except that treatment with sodium hydroxide was performed only once and the DNA was dissolved in 0.3 ml of 0.2 N ammonia, and the radioactivity was determined in a liquid scintillation counter. The reaction mixture contained, per ml: 40 μmol of Tris-hydrochloride, pH 8; 16 μmol of 2-mercaptoethanol; 0.8 μmol of EDTA; 40 nmol of S-adenosyl[methyl-^{14}C]methionine (specific activity, 53 Ci/mol); 48 μg of E. coli B DNA; and 40 to 80 μg of enzyme protein. Incubations were for 30 min at 37°C. In some experiments the methylated bases were identified as previously described (9). Less than 2% of the radioactivity incorporated into 5-methylcytosine was incorporated into N^6-methyladenine with extracts of E. coli B, or K-12 derivatives, containing the plasmid-specified EcoRII methylase. Ten percent of the radioactivity was incorporated into N^6-methyladenine with extracts of mec[+] K-12 strains not containing these plasmids.

Enzyme inhibition studies were performed by incubating partially purified enzyme prepared from E. coli B(R15) (10) with 0.01 ml of cell extract for 5 min at 37°C in a 0.2-ml reaction mixture before the addition of E. coli B DNA and S-adenosylmethionine as described above. Protein was determined by the method of Lowry et al. (16).

RESULTS

Effect of 5-azacytidine on cell growth. 5-Azacytidine at 20 μg/ml inhibited growth of E. coli B, but this inhibition was completely reversible (Fig. 1). When an EcoRII restriction-modification system was placed in these cells, their response to 5-azacytidine changed. Recovery was much slower than with cells not containing the plasmid, and after 1 h growth ceased. Greater than 98% of the cells were killed within 15 min (Fig. 2) of addition of 5-azacytidine (20 μg/ml) to the growing cells. E. coli B was resistant to this effect of 5-azacytidine; even at 1 mg of azacytidine per ml, no bactericidal effect was seen (data not shown).
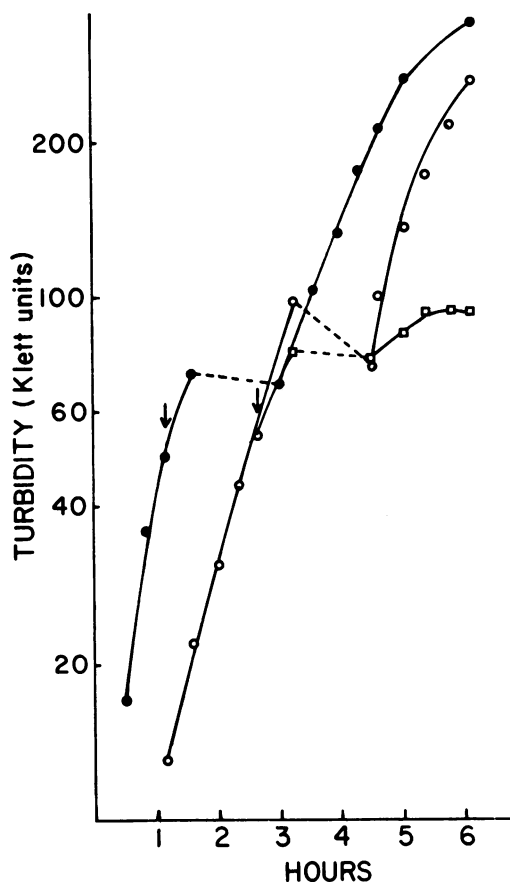
Effect of base analogs on the growth of E. coli B

teria containing plasmids that did not specify such enzymes (pBR322, 459-S2), or that contained a plasmid that had lost this specificity (390-S2), were not killed by 5-azacytidine. The effect occurred not only in *E. coli* B; F1100, a substrain of *E. coli* K-12, was also killed by 5-azacytidine if it contained a plasmid specifying *Eco*RII restriction-modification enzymes (Table 3).

Deamination of 5-azacytidine to 5-azauridine was not required for cell death, as has been suggested for its inhibition of cell growth (8), since it was bactericidal to strain Sφ441(N3), which lacks the enzyme cytidine deaminase. The cells had to be growing for the bactericidal effect of the drug to be observed; stationary-phase cells were not killed by the drug.

Effect of azacytidine on *Eco*RII restriction-modification mutants. *E. coli* B containing the mutant plasmid deficient in restriction responded to azacytidine just as did strains containing the wild-type plasmid (Table 3). This was also true of three restriction mutants of plasmid R15 isolated by us (data not shown).

A modification mutant, supplied by S. Hatt-

FIG. 1. Effect of 5-azacytidine on the growth of *E. coli*. *E. coli* B (●) and *E. coli* B(R15) (○, □) were grown in minimal medium containing 1% Casamino Acids. At the indicated time (arrows), 20 μg of 5-azacytidine per ml was added (●, □), and the cells were incubated for 30 min. The cells were washed twice, suspended in culture medium and reincubated. ○, Untreated *E. coli* B(R15), control.

and *E. coli* B(R15). None of the other base analogs tested had as dramatic an effect on the growth of *E. coli* B(R15) as did 5-azacytidine (Table 2). Two other cytidine analogs, 5-fluoro-cytidine and ψ-isocytidine, also inhibited growth of *E. coli* B(R15) to a greater extent than *E. coli* B. ψ-Isocytidine caused a 10% decrease in viable cells. None of the other drugs listed in Table 2 caused a decrease in viable cells of *E. coli* B(R15), nor did 5-fluorouracil, 6-azacytidine, 5-azacytosine, or 5-fluorouridine, although most of them did inhibit growth.

Effect of 5-azacytidine on strains other than *E. coli* B[R15 Res$^+$ Mod$^+$ (RII)]. The effect of 5-azacytidine on the growth of *E. coli* was limited to those strains harboring plasmids containing *Eco*RII restriction-modification enzymes. Bac-
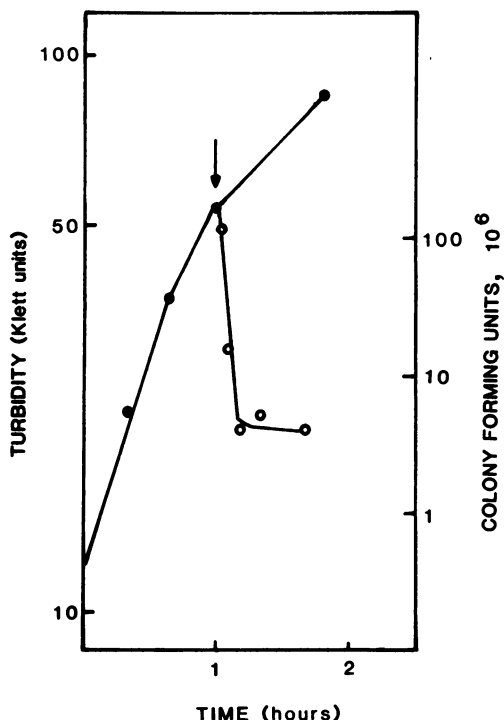

FIG. 2. Effect of 5-azacytidine on growth of *E. coli* B(R15). *E. coli* B(R15) was grown in minimal medium containing 1% Casamino Acids; at the indicated time (arrow), 20 μg of 5-azacytidine per ml was added. Equal portions were removed, and viable counts (○) were determined.

TABLE 2. Effect of base analogs on growth[a]

Drug	Concn µg/ml	CFU (% of control)[b]	
		E. coli B(R15)	E. coli B
5-Azacytidine	20	0.3	55
5-Azacytidine + cytidine	20 100	89	
5-Azauracil	333	60	61
6-Azauridine	333	82	68
ψ-Isocytidine	400	38	89
5-Fluorocytidine	333	68	83

[a] E. coli was grown to 50 to 55 Klett units of turbidity and then grown for 30 min in the presence or absence of the base analog. Colony-forming units were then determined. Each determination is the average of at least two experiments.

[b] For E. coli B and E. coli B(R15), respectively, colony-forming units (CFU) for the controls were: at zero time, $1.3 \pm 0.5 \times 10^8$ and $1.4 \pm 0.7 \times 10^8$; and at 30 min, $2.7 \pm 0.4 \times 10^8$ and $2.2 \pm 0.6 \times 10^8$.

TABLE 3. Effect of 5-azacytidine on cell survival[a]

E. coli strain	Colony-forming units (10^8 cells/ml)		Methylase activity[b] (pmol/min per mg of protein)
	0^c	30	
B	1.0	1.5	<0.1
K-12	0.9	2.5	0.7
K-12 (pBR322)	0.8	3.0	
B(R15)	2.3	0.02	14.5
B(N3)	0.6	0.003	16.7
B(R390-S1)	1.3	<0.001	
B(R390-S2)	1.2	2.9	
B[N3 Res⁻ Mod⁺ (RII)]	2.2	0.006	17.4
B[R15 Res⁻ Mod⁺ (RII)]	2.3	0.02	16.6
B(R459-S2)	1.9	2.4	
B[N3 Res⁻ Mod(Am) (RII)]	1.1	1.0	0.1
F1100(N3)	0.8	0.008	7.9
F1100 mec[N3 Res⁻ Mod⁺ (RII)]	1.9	0.003	7.5
F1100 mec[N3 Res⁻ Mod(Am) (RII)]	2.1	0.9	2.5
F1100 mec	2.1	2.3	0.1
Sϕ441	1.0	1.0	
Sϕ441(N3)	2.0	0.005	
B(R15)[d]	42	36	

[a] E. coli strains were treated as described in Table 2.
[b] Methylase activity of extracts of untreated cells.
[c] Time after addition of 5-azacytidine (20 µg/ml).
[d] Cells were in stationary phase.

man (23), was found to be relatively resistant to the bactericidal effect of 5-azacytidine in E. coli B, although cells containing the plasmid were more sensitive to the drug than were cells lacking it. This methylase mutant is an amber-suppressible mutant, and the effect of 5-azacytidine on cells containing this mutation was also tested in F1100 mec, a suppressing strain. F1100 mec [N3 Res⁻ Mod(Am) (RII)] was more resistant to 5-azacytidine than were strains containing either the wild-type plasmid or an N3 Res⁻ Mod⁺ (RII) plasmid, but more sensitive than F1100 mec itself. E. coli K-12 contains a cytosine methylase which has the same specificity as the EcoRII methylase (12, 13), but K-12 strains were resistant to the bactericidal effect of 5-azacytidine.

Methylase levels in E. coli strains. As shown by May and Hattman (18) and confirmed here, E. coli K-12 had very low methylase levels compared with strains carrying plasmids that specify EcoRII restriction-modification enzymes, and E. coli B containing the Res⁻ Mod(Am) (RII) plasmid had barely detectable levels of methylase—less than 1% of the activity in strains carrying the wild-type plasmid. F1100 mec Su⁺ carrying this plasmid had methylase levels that were one-third the level in strains carrying Res⁺ Mod⁺ (RII) or Res⁻ Mod⁺ (RII) plasmids (Table 3). These strains were also tested for the ability to modify phage by measuring the EOP on strains carrying the EcoRII restriction-modification system (Table 4). The EOP of T1·B[N3 Res⁻ Mod(Am) (RII)] was approximately the same as T1·B; the EOP of T1·F1100 mec[N3 Res⁻ Mod(Am) (RII)] was greater than that of T1·F1100 mec but less than that of

T1·F1100(N3). With phage λ, however, the efficiency of plating of λ·F1100(N3) and λ·F1100 mec[N3 Res⁻ Mod(Am) (RII)] was approximately the same. The N3 system appeared to be more efficient in modifying λ than T1 (Table 4). The failure of F1100 mec to completely suppress the amber mutation could therefore be detected with T1 phage but not with λ.

Effect of inhibition of DNA synthesis on the killing effect of 5-azacytidine. Presumably, 5-

TABLE 4. EOP of T1 and λ phage[a]

Phage	EOP
T1·B(N3)	0.38
T1·B	1.2×10^{-4}
T1·B[N3 Res⁻ Mod(Am) (RII)]	8.4×10^{-5}
T1·F1100(N3)	0.15
T1·F1100 mec	0.005
T1·F1100 mec[N3 Res⁻ Mod(Am) (RII)]	0.03
λ·F1100(N3)	1.0
λ·F1100 mec	2.6×10^{-4}
λ·F1100 mec[N3 Res⁻ Mod(Am) (RII)]	0.81

[a] T1 phage was plated on E. coli B and E. coli B(N3) or on F1100 mec and F1100(N3). Lambda phage was plated on F1100 mec and F1100(N3).

TABLE 5. Effects of nalidixic acid and coumermycin on 5-azacytidine-induced cell death and methylase activity[a]

Treatment	CFU (10^8)	Enzyme activity (pmol/min per mg of protein)
None	2.9	12.0 ± 1.3[b] (5)[c]
5-Azacytidine	0.02	1.6 ± 1.1 (4)
Nalidixic acid	0.9	7.9 (1)
Nalidixic acid + 5-azacytidine	0.01	2.8 ± 1.5 (4)
Chloramphenicol	1.4	
Chloramphenicol + 5-azacytidine	0.02	1.0 (2)
Coumermycin	0.5	8.7 (1)
Coumermycin + 5-azacytidine	0.2	4.9 ± 1.2 (3)
Novobiocin	0.9	
Novobiocin + 5-azacytidine	0.15	

[a] *E. coli* B(R15) was grown to 50 to 55 Klett units of turbidity; portions were treated with 50 μg of antibiotic per ml, except for novobiocin, which was used at 500 μg/ml, for 10 min; 20 μg of azacytidine per ml was then added, and colony-forming units (CFU) or enzyme concentration was determined 20 min later.

[b] Standard deviation.

[c] Number of determinations.

azacytidine kills *E. coli* B(R15) because it inhibits the DNA (cytosine-5)methylase present in these cells. Since in vitro this enzyme is inhibited by 5-azacytosine-containing DNA, we tested several inhibitors of DNA synthesis for their ability to inhibit the killing effect of 5-azacytidine. Coumermycin partially blocked the killing effect of 5-azacytidine; more cells survived the combined treatment than survived treatment with 5-azacytidine alone, even though coumermycin killed 70% of the cells. Novobiocin also protected the cells from the bactericidal effect of 5-azacytidine, but it was not as effective as coumermycin. Pretreatment with nalidixic acid did not protect against 5-azacytidine (Table 5). Incorporation of thymidine was completely inhibited by the dose of nalidixic acid used. Chloramphenicol did not protect cells against the effects of 5-azacytidine.

I determined whether there was any correlation between the ability of these drugs to inhibit the killing effect of azacytidine and their ability to decrease the inhibition of the DNA methylase caused by 5-azacytidine. Both coumermycin and nalidixic acid decreased the enzyme inhibition caused by 5-azacytidine, but treatment with coumermycin afforded better protection than did treatment with nalidixic acid (Table 5).

Extracts of cells treated with azacytidine alone, or chloramphenicol plus azacytidine, contained an inhibitor of the enzyme (Table 6). This inhibitor could be completely destroyed by incubating the extract with pancreatic DNase. No such inhibitor could be detected in cells that had been pretreated with either nalidixic acid or coumermycin, although the methylase activity was lower in these extracts than in extracts of control cells (Table 5). The synthesis of the inhibitor in cells exposed to 5-azacytidine was independent of the presence of plasmids specifying *Eco*RII systems because extracts prepared from 5-azacytidine-treated *E. coli* B cells also inhibited the methylase (Table 6).

Addition of crude extract to purified enzyme always caused an increase in the amount of product formed, even when the crude extract came from *E. coli* B, which has no DNA cytosine methylase of its own. This stimulatory effect was also seen with the extracts obtained from cells treated with 5-azacytidine and either coumermycin or nalidixic acid.

DISCUSSION

Treatment with 5-azacytidine was lethal to cells containing plasmids specifying *Eco*RII restriction-modification systems but not cells lack-

TABLE 6. Effect of drug treatment on the synthesis of DNA methylase inhibitor[a]

Strain	Treatment	Enzyme activity (pmol/min)	
		Extract	Extract + methylase
B(R15)	Chloramphenicol + 5-azacytidine	0.01	0.14
B(R15)	Nalidixic acid + 5-azacytidine	0.08	0.39
B(R15)	Coumermycin + 5-azacytidine	0.12	0.45
B(R15)	AzaCyd	0.04	0.08
B	None	<0.01	0.90
B[b]	5-Azacytidine + DNase		0.80
B[c]	5-Azacytidine		0.04

[a] *E. coli* cells were grown and treated as described in Table 5. Enzyme extracts were prepared from 25 ml of cells as described in Materials and Methods. The extract, 10 μl of protein (1 to 2 mg/ml), was assayed alone or with the addition of partially purified *E. coli* RII methylase, 0.23 or 0.58 pmol/min of added activity, respectively, in the upper and lower sections of the table.

[b] The extract was preincubated for 30 min with 50 μg of pancreatic DNase per ml in the presence of 5 mM MgCl$_2$ before addition of purified enzyme and 10 mM EDTA.

[c] The extract was treated as in *b*, but without addition of pancreatic DNase.

ing these systems. No other pyrimidine analog tested had as marked an effect on these cells. The effect of 5-azacytidine is not instantaneous: it takes 15 min for the full effect to be seen (Fig. 2). Furthermore, the cells must be growing for the bactericidal effect to occur; cells in stationary phase are resistant to the drug (Table 3). These findings are consistent with the inhibitor not being 5-azacytidine itself, but either 5-azacytosine in DNA or a metabolite of the drug.

The lethal effect is due to the presence of the EcoRII restriction-modification system in the cell, since plasmids not containing this system, or that have lost it due to deletion, do not confer sensitivity to the drug. It does not occur in cells having restriction-modification systems dependent on adenine methylation, since E. coli strains K-12 and B both contain such systems (22).

Sensitivity to the drug is not a function of the presence of the restriction enzyme, since mutants lacking this enzyme are still sensitive to the drug (Table 3). Sensitivity is a nonlinear correlate of the EcoRII methylase activity. A mutant deficient in this enzyme is much less sensitive to the drug than cells containing the enzyme (Table 3). The mutation is an amber mutation, and a strain carrying a suppressor, E. coli F1100 mec, which expresses 30% of the wild-type enzyme (Table 3), is relatively insensitive to the effects of 5-azacytidine. Furthermore, K-12 strains, which contain 10 to 20% of the methylase activity present in plasmid-containing strains, are resistant to the bactericidal effects of this drug.

Since the methylase is not necessary for cell viability, it is surprising that inhibition of this enzyme should result in cell death. One plausible explanation, consistent with my earlier findings on the mechanism of inhibition of the methylase, is that the enzyme reacts irreversibly with azacytosine-containing DNA, inhibiting further DNA and RNA synthesis. Drugs that inhibit DNA synthesis were tested to determine whether they would prevent the lethal effect of 5-azacytidine. Coumermycin inhibited the lethal effect of 5-azacytidine, novobiocin partially blocked the killing effect, and nalidixic acid had no detectable effect at a concentration that completely inhibited thymidine incorporation. Consistent with these results is that coumermycin prevented the inhibition of the methylase by azacytidine to a greater extent than did nalidixic acid (Table 5). Since nalidixic acid did not prevent the bactericidal effect of 5-azacytidine, we cannot at present be certain that the proposed mechanism is correct.

The current experiments indicate that there is some function of the methylase, in cells carrying plasmids that specify the EcoRII enzyme, that sensitizes these cells to 5-azacytidine. We shall have to isolate additional methylase mutants to ascertain the relationship between the function of the methylase and the bactericidal effect of the drug. These experiments do, however, indicate that a drug that inhibits a superfluous enzyme system, which does not metabolize that drug to an active metabolite, can cause cell death.

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