VIROLOGY 13, 493–499 (1961)

Effect of Mitomycin C on Interactions between Temperate Phages and Bacteria[1]

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Accepted December 21, 1960

DNA synthesis is selectively inhibited in *Salmonella typhimurium* by treatment with the antibiotic mitomycin C. These inhibited cells will, however, produce active particles on infection with phage P22. Genetic recombination between marked phages is not affected, but the frequency of lysogenization is drastically reduced under these conditions. The transducing activity of phage P22 lysates formed in the presence of the antibiotic is also lowered. Mitomycin C mimics the activity of ultraviolet in causing the induction of phage production in some lysogenic strains but not in others.

INTRODUCTION

The antibiotic mitomycin C (MC) selectively inhibits the synthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* B; ribonucleic acid (RNA) and protein formation are not affected under appropriate conditions (Shiba *et al.*, 1958, 1959; Sekiguchi and Takagi, 1959, 1960). DNA synthesis can be restored in inhibited cells by infection with the virulent bacteriophages T2, T3, and T5. The restored synthesis results in the production of mature phage (Sekiguchi and Takagi, 1959, 1960). MC also induces phage production in *E. coli* K12 cells lysogenic for λ prophage (Otsuji *et al.*, 1959).

This paper extends the earlier reports by examining the effect of MC on a number of interactions between temperate phages and bacteria. The results of treatments with the antibiotic on DNA synthesis in *Salmonella typhimurium*; on phage production, phage recombination, and on the establishment of lysogeny in cells infected with phage P22; and on transducing activity of phage P22 are presented. Last, a comparison of induction of phage production in lysogenic bacteria by MC and ultraviolet (UV) light is reported.

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains

Phage P22, its host *S. typhimurium* LT-2, some mutants of strain LT-2, and a derivative lysogenic for P22 are employed in some of these experiments. In addition, *E. coli* C lysogenic for the prophage of phage P2*c* and its indicator, a phage-sensitive, streptomycin-resistant mutant of *E. coli* C, are used in induction experiments.

The wild-type temperate phage P22 c^+ and a virulent mutant c_2 are used. Bacteria infected with phage c_2 do not give rise to lysogenic progeny; all infected cells lyse and liberate progeny phages (Levine, 1957). The plaque morphology markers m_3 and h_{21} (Levine, 1957) are used in genetic recombination experiments. These are scored on indicator agar (Bresch, 1953).

Media

The following media are utilized: L broth, L agar, nutrient broth, EMB galactose agar, indicator agar, soft agar for top layers, and buffered saline. The preparation of these media has been described (Levine, 1957). In addition, Davis' medium (7 g

K_2HPO_4, 2 g KH_2PO_4, 0.5 g Na citrate. $5H_20$, 1 g $(NH_4)_2SO_4$, and 0.1 g $MgSO_4$ in 1 liter of distilled H_2O; 0.1% glucose added after autoclaving; for plates 1.5% agar and a trace of tryptone are added) is used as a minimal medium.

Mitomycin C

Crystalline mitomycin C was supplied through the kindness of Dr. J. Lein of the Bristol Laboratories and Dr. L. D. Hamilton of the Sloan-Kettering Institute. Stock solutions are made in sterile distilled water at a concentration of 1000 μg/ml.

Experimental Procedures

Protein determination. The protein content of bacterial cultures is determined by the Folin reaction according to the procedure of Lowry *et al.* (1951).

RNA determination. One milliliter of cells is added to 3 ml of cold 0.5 *M* perchloric acid and centrifuged. The pellet is resuspended in 1.5 ml $FeCl_3$-HCl reagent (0.1% $FeCl_3$ in concentrated HCl) and 0.15 ml orcinol reagent [100 mg/ml in 95% ethanol (Mejbaum, 1939)]. The mixture is placed in boiling water for 30 minutes, then cooled rapidly and read at 670 mμ.

TABLE 1

SELECTIVE INHIBITION OF DNA SYNTHESIS IN *Salmonella Typhimurium* BY MITOMYCIN C[a]

Concentration of MC (μg/ml)	Time of incubation (min)	Turbidity 490 mμ	Protein (Folin) 750 mμ	RNA (orcinol) 670 mμ	DNA (diphenyl amine) 595–650 mμ
0	0	0.258	0.111	0.220	0.045
0	30	0.409	0.139	0.336	0.052
1	30	0.420	0.149	0.385	0.057
5	30	0.405	0.142	0.363	0.049
10	30	0.405	0.162	0.357	0.045
0	60	0.610	0.206	0.579	0.099
1	60	0.580	0.220	0.570	0.084
5	60	0.565	0.203	0.578	0.071
10	60	0.480	0.180	0.471	0.047

[a] The values in columns 3–6 are given in optical density units as determined with the Beckman model B spectrophotometer.

DNA determination. Two milliliters of cells is added to 2 ml of 0.5 *M* cold perchloric acid and centrifuged. The pellet is washed once with 0.2 *M* perchloric acid and resuspended in 1 ml of 0.5 *M* perchloric acid. Two milliliters diphenylamine reagent [1.0 g diphenylamine in 100 ml glacial acetic acid plus 2.75 ml concentrated H_2SO_4 (Dische, 1930)] is added and the color is allowed to develop for 18 hours at 30°. The difference in optical density at 595 mμ and 650 mμ is then determined.

Turbidity of bacterial cultures is measured by changes in optical density at 490 mμ. All optical density readings are carried out in the Beckman model B spectrophotometer. Other experimental methods will be described as needed.

RESULTS

Inhibition of DNA Synthesis in LT-2 Cells. Logarithmic phase bacteria (3×10^8 cells/ml) growing in Davis' medium are exposed to various levels of MC at 37° with aeration. Samples are removed after 30- and 60-minute treatment periods, washed twice by centrifugation with buffered saline, and resuspended in buffered saline. These samples are used for turbidity, DNA, RNA, and protein determinations.

MC treatment selectively inhibits DNA synthesis in uninfected strain LT-2 cells (Table 1) as it does in *E. coli* B. Complete inhibition is achieved by exposure to 10 μg/ml of MC. RNA and protein formation are maintained at control levels for the first 30 minutes of treatment, but fall behind untreated cells during the second 30 minutes. Immediate inhibition of all three components is found at 20 μg/ml of the antibiotic. Partial inhibition of DNA synthesis occurs with 5 μg/ml of MC, but 1 μg/ml does not inhibit for at least the first 30 minutes of treatment. Similar results are obtained when cells are exposed to MC in L broth.

The observed increase in turbidity of treated cultures is due to cell enlargement. No evidence for cell multiplication, as measured by increase in number of colony formers, is found with any MC treatment. In fact, all concentrations show some bactericidal action; 89% of the treated cells

survive 60 minutes at 1 μg/ml, 27% at 5 μg/ml, and only 4% at 10 μg/ml. The DNA content of cells treated at 1 μg/ml almost doubles in 60 minutes, suggesting that MC has an effect on cell division in addition to the more obvious one of inhibiting DNA synthesis.

Phage production in MC-treated sensitive cells. As with phages T2, T3, and T5, and *E. coli* B, DNA-inhibited strain LT-2 cells produce phage progeny when infected with either phage c_2 or phage c^+. Phage production is taken to indicate restoration of DNA synthesis.

Bacteria at a concentration of 10^8 cells/ml in L broth are washed twice in buffered saline and are aerated for 45 minutes at 37°. They are then exposed to a suspension of phage c_2 or c^+ sufficient to give a multiplicity of infection of 10. After a 5 minute adsorption period, a 1:10 dilution is made into L broth containing 10 μg MC/ml and antiphage serum in concentration capable of inactivating 99% of the unadsorbed phage in 5 minutes. MC does not interfere with antiserum activity. A further dilution is then made into L broth containing 10 μg MC/ml. At various times during the latent period a further dilution into L broth is made to remove MC, and samples are plated on EMB galactose agar plates with gal^--sensitive bacteria as indicator. Plating on EMB galactose agar plates permits the detection of infective centers and surviving bacteria which may or may not be lysogenic (Levine, 1957).

Infectious phage particles are produced by all treated cells, whether infected with phage c_2 or phage c^+. No loss of infective centers takes place in samples removed from the antibiotic at any time during the latent period. The duration of the latent period is the same (32 minutes) for cells that lyse in MC and for untreated controls. The yield of progeny particles per infected cell is, however, about 50% that of controls: 157 as compared to 350 in a typical experiment.

The establishment of lysogeny is inhibited by the presence of MC. Exposure of phage c^+ infected cells to 10 μg/ml of the antibiotic for as short a time as 5 minutes during the latent period causes a shift from the lysogenic to the lytic response (Table 2). All treated cells lyse, while about 90% of the untreated bacteria give rise to lysogenic progeny.

TABLE 2

FREQUENCY OF LYSOGENIZATION FOLLOWING MITOMYCIN C TREATMENT OF PHAGE c^+-INFECTED CELLS

Concentration of MC (μg/ml)	% Lysogenization after treatment for		
	5 min	10 min	15 min
0	91	—	—
1	83	53	58
5	—	0	—
10	0	0	0

Genetic recombination between mutant phages is not affected by MC. Crosses between appropriately marked c_2 phages ($m_3\ c_2\ h_{21} \times +c_2+$) carried out in 10 μg/ml of the antibiotic show recombination frequencies that are not significantly different from those obtained in control crosses.

Induction of phage production in lysogenic bacteria. Lysogenic bacteria are, as a rule, stable and show rates of spontaneous lysis characteristic for each system. As an example, only one cell in 10,000 per division cycle spontaneously produces phage in cultures of strain LT-2 lysogenic for phage P22. The shift to vegetative reproduction may be enhanced in "inducible" systems by a number of treatments, including exposure to UV irradiation (Lwoff *et al.*, 1950). Some prophages are "noninducible" by these treatments. The λ prophage is induced by both UV light and MC (Otsuji *et al.*, 1959). The question arises whether MC can induce phage production in a lysogenic system that is not induced by UV. To test this point two other lysogenic bacteria were exposed to the antibiotic. One is UV inducible strain LT-2 carrying P22 c^+; the other, *E. coli* C carrying P2*c*, is noninducible by UV light (Bertani, 1958).

The induction experiments are carried out in the following way. Log-phase lysogenic LT-2 cells are exposed to different concentrations of MC in L broth. After 5, 10, and 15 minutes of incubation at 37°, samples are diluted in fresh L broth and are plated with gal^--sensitive bacteria on EMB ga-

lactose agar. Plaque formation on these plates is indicative of induction. For phage P2 induction, lysogenic *E. coli* C cells are exposed to either 10 μg/ml or 20 μg/ml of the antibiotic in nutrient broth for 10 minutes. The cells are then centrifuged and resuspended in fresh nutrient broth containing anti-P2 serum to inactivate most of the free phages. The cultures are diluted 1:3 and then plated with streptomycin-resistant plating bacteria on L agar plates. These plates are incubated at 37° and at intervals are sprayed with a solution of dihydrostreptomycin. The streptomycin kills the lysogenic cells on the plates, but each cell that has lysed or is lysing gives rise to a plaque on the streptomycin-resistant indicator (Six, 1959). (This experiment was carried out with the kind collaboration of Dr. G. Bertani.)

Induction of phage P22 occurs in significant amounts following MC treatments, which also decrease survival significantly: 5 μg/ml or higher (Table 3). An exposure to 10 μg/ml for 10 minutes, giving 45% survival, induces 68% of the surviving cells. The yield of phage particles from the induced cells is low, averaging about 30 particles per cell. Cells lysogenic for phage P2, on the other hand, show no increased induction following a 10-minute treatment with either 10 μg/ml or 20 μg/ml, despite the considerable inactivation of cells achieved at these doses. Preliminary experiments indicate that the prophage of phage P1 (carried in *E. coli* B) can also be induced by MC treatment. This system is slightly inducible with UV light. It appears that MC mimics the inducing activity of UV light.

TABLE 3

FREQUENCIES OF INDUCTION AND SURVIVAL FOLLOWING MITOMYCIN C TREATMENT OF LYSOGENIC LT-2 CELLS

Concentration of MC (μg/ml)	% Induction and survival after treatment for					
	5 min		10 min		15 min	
	I[a]	S[b]	I	S	I	S
0	0	100	0	100	0	100
0.1	3	100	1	100	5	100
1.0	7	100	6	100	6	75
5.0	13	57	41	43	46	64
10.0	29	39	68	45	60	30
20.0	79	22	73	18	91	11

[a] I = induction. The per cent induction for a given treatment is calculated from the number of plaques divided by the total number of plaques and colonies found on the EMB plates multiplied by 100.

[b] S = survival. The frequency of survivors is calculated from the total number of plaques and colonies obtained after treatment divided by the total number found in controls not treated with MC multiplied by 100.

Another antibiotic, phagolessin A58, also mimics UV light, inducing two UV-inducible lysogenic strains and not affecting two others which are not inducible (Hall-Asheshov and Asheshov, 1956).

Transduction by phage produced in the presence of MC. Transduction of bacterial characters by phage P22 is a well-studied phenomenon (Zinder, 1953; Hartman, 1957). Phage P22 produced in MC-inhibited bacteria exhibits markedly decreased transducing activities. Such particles are prepared by infection of wild-type strain LT-2 cells treated with 10 μg/ml of the antibiotic in L broth for 15 minutes before infection and kept in it until lysis is completed. The particles are washed clean of the antibiotic by centrifugation and are resuspended in buffered saline. Two mutant strains, one *gal*$^-$ and the other requiring histidine (*his*$^-$ 22, provided by Dr. P. E. Hartman) are used as recipient bacteria. *Gal*$^+$ transductants are detected by plating on EMB galactose agar, and *his*$^+$ transductants by plating on M9 minimal plates.

The transducing activities of three independently prepared MC lysates are compared with those of three normal lysates. The transducing frequencies of the MC lysates are, at most, one-tenth that of the normal lysates for both the galactose and the histidine markers. This is true for each of the wide range of multiplicities of infection used. In one experiment, the transducing activity of the treated lysate is only one-fiftieth that of the normal.

DISCUSSION

The antibiotic activity of MC is due to inhibition of DNA synthesis. The inhibitory

mechanism is unknown, but DNA synthesis is restored by phage infection. The reversal of inhibition by infection with phage T2 might suggest a relation with the occurrence of the unique base 5-hydroxymethylcytosine in its DNA (Wyatt and Cohen, 1952), implicating cytosine metabolism as the site of inhibition. However, the DNA's of phages T3, T5, and P22 contain cytosine rather than 5-hydroxymethylcytosine; yet, infection with these phages also restores DNA synthesis. Clearly, cytosine cannot be the critical site.

Recovery of the ability to synthesize DNA following phage infection is, of course, not limited to MC treatment. Bacteria treated with UV irradiation (Watanabe *et al.*, 1952) and thymine-requiring bacteria deprived of thymine (Barner and Cohen, 1954) synthesize DNA and produce phage upon infection. An explanation commonly offered for these findings is that the newly introduced viral DNA replaces the damaged host genetic material, thus repairing the functional lesions. It should be stressed that in the case of MC treatment, synthesis of phage DNA occurs in the continued presence of inhibitor. On the one hand, this suggests that infection induces a specific (alternate) pathway for phage DNA formation. On the other hand, it may be supposed that MC blocks DNA synthesis in an early step, perhaps in the formation of one of the deoxynucleotides and that phage DNA is synthesized in the presence of MC from preformed components of bacterial DNA. Lower yields of particles might then be expected and are, indeed, observed. MC-treated strain LT-2 cells produce only about half as many particles as are produced by untreated cells. The decrease in transducing activity of phage lysates prepared in the presence of the antibiotic might be a consequence of extensive degradation of bacterial DNA to low molecular weight constituents incapable of carrying genetic information. Sekiguchi and Takagi (1960) report that acid-soluble deoxyriboside compounds accumulate in MC-inhibited cells. Whether these represent precursors that fail to polymerize or degradation products of DNA is not known.

MC joins a long list of agents that induce lysogenic bacteria, all of which probably affect bacterial DNA synthesis. Recently, Melechen and Skaar (1960) reported induction of phage P1 in a P1-lysogenic, thymine-requiring mutant of *E. coli* B as a consequence of thymine deprivation. Another inhibitor of DNA synthesis, 5-fluorodeoxyuridine (Cohen *et al.*, 1958), appears to induce both phage P22 and phage λ (Levine, unpublished).

Jacob and Campbell (1959) have postulated the formation, under the control of prophage, of specific cytoplasmic "immunity" substances that act as repressors of vegetative phage production. The chemical nature of the immunity substance is unknown, but it appears *not* be to a protein. The experiments of Bertani (1957) and Christensen (1957) show that treatment with chloramphenicol of cells infected with temperate phage causes a shift from the lytic to the lysogenic response. Chloramphenicol is an inhibitor of protein synthesis. On the other hand, the initiation of induction as a result of treatments that inhibit bacterial DNA synthesis, such as MC treatment, suggests the immunity substance may itself be DNA or may require DNA synthesis to be produced. It is conceivable, however, that cessation of bacterial DNA synthesis upsets the normal relationship between cell and prophage, stopping the production of immunity substance, which, on this view, need not be DNA.

Ultraviolet irradiation and MC treatment produce a number of similar effects: (1) Both inhibit DNA synthesis in bacterial cells. (2) In both cases, DNA synthesis can be restored by phage infection. (3) Either agent causes a shift from lysogeny to lysis in cells infected with a temperate phage. (4) MC induces lysogenic bacteria that are also inducible by UV light. (5) Pretreatment of lysogenic cells with chloramphenicol protects against induction by both UV light and MC (Levine, unpublished). (6) Phages λ and T3 inactivated by UV light or by nitrous acid can be reactivated upon infecting bacteria treated with MC or with UV light (Otsuji and Okubo, 1960). It should, however, be pointed out that MC does not mimic the effects of UV light in all ways: (1) There is no evidence that MC

treatment causes damage that can be reactivated by visible light. At least part of the UV damage can be photoreactivated (Kelner, 1953). (2) Ultraviolet irradiation of mixedly infected bacteria results in increases in the frequency of phage recombination (Jacob and Wollman, 1955). No such increase is found with MC treatment. (3) The adaptive formation of β-galactosidase in *E. coli* B is strikingly inhibited by UV irradiation at doses that inhibit DNA synthesis (Torriani, 1956). MC concentrations sufficient to inhibit DNA synthesis completely do not affect β-galactosidase formation (Shiba *et al.*, 1958).

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