

Enhancement of *Agrobacterium tumefaciens* Infectivity by Mitomycin C

GARY T. HEBERLEIN¹ AND JAMES A. LIPPINCOTT

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

Received for publication 26 July 1967

The ability of *Agrobacterium tumefaciens* to induce pinto leaf tumors may be enhanced two- to threefold after treatment with mitomycin C. The enhancement may be obtained with either lethal or nonlethal concentrations. With 10-min treatments, an optimal response was obtained with 0.005 μg of mitomycin C per ml in the absence of any change in the number of viable cells. Both the tumor induction process and the tumors induced by treated cultures appear qualitatively the same as controls. To account for these results, the antibiotic must increase the proportion of viable cells that will subsequently initiate tumors. One, or at most a few, random lesions in the bacterial chromosome seem to be the necessary requirement for this promotion. At mitomycin concentrations of 1 and 5 $\mu\text{g}/\text{ml}$, the ability of *A. tumefaciens* to initiate tumors is rapidly lost, indicating that a fairly intact bacterial chromosome is one of the essentials for the tumor induction process.

Bacteria treated with the antibiotic mitomycin C (MC) show a spectrum of responses similar to those obtained by irradiating bacteria with short wavelength ultraviolet light. Inhibition of deoxyribonucleic acid (DNA) synthesis (2, 12, 22-24), mutation (8, 25), prophage development (4, 12, 19, 20, 29), bacteriocin production (17), and bacterial filament formation (5, 11, 22; H. Suzuki and W. W. Kilgore, *Bacteriol. Proc.*, p. 40, 1964) may be induced by MC. The activity of this antibiotic results from its participation incross-links formed between the two complementary DNA strands of the treated organism (9, 10, 18, 26).

The ability of *Agrobacterium tumefaciens* to induce tumors may be enhanced by irradiation with short wavelength ultraviolet light (6, 7). Because of the action of blue light in preventing this promotion under certain conditions, it was proposed that lesions induced in the DNA of the bacterium were necessary for this promotion. This contention would be greatly strengthened if MC were shown to induce similar changes in the infectivity of *A. tumefaciens*.

The results presented below show that MC treatment can produce an enhancement of the tumor-inducing ability of *A. tumefaciens* comparable to that obtained with ultraviolet light. The infectivity promotion may be obtained under conditions resulting in no loss in bacterial via-

bility, indicating that a greater portion of the cells in a treated culture sample subsequently participate in tumor initiation than in the control.

MATERIALS AND METHODS

Bacteria. Strain B6 of *A. tumefaciens* (Smith and Town) Conn was used in these experiments. The growth media and culturing conditions employed have been described (6, 7, 13). For most experiments, cultures were grown to late log phase (18 hr) from an inoculum of ca. 10^7 bacteria per ml. The number of viable cells in control and MC-treated cultures was estimated by plating in triplicate one or more concentrations of a $10\times$ serial dilution series.

MC treatments. The entire contents of a vial of MC (Calbiochem, Los Angeles, Calif.) were dissolved in a known volume of distilled water and stored in a deep freeze. Dilutions from this stock were made at regular intervals for current experiments. The MC treatments consisted of adding 2-ml portions of appropriate dilutions of the antibiotic to 18 ml of a bacterial culture at room temperature (ca. 25 C). Control samples of the culture (18 ml) received 2 ml of distilled water. Following this addition, control and treated cultures were stirred on a magnetic stirrer, and test samples were removed at various times for viability and infectivity determinations. The reaction was "stopped" by a 10-fold dilution of the samples with cold distilled water immediately after removing the sample from the reaction flask. The tubes containing the diluted samples were supported in crushed ice until used for inoculation onto pinto bean leaves and for further dilution for viability determinations. These storage and dilution conditions

¹Present address: Department of Biology, New York University, Bronx, N.Y.

had no effect on the viability and infectivity determinations.

Infectivity determinations. The infectivity of control and MC-treated samples was determined by inoculating each sample on 12 to 16 primary pinto bean leaves following the quantitative bioassay procedure described for this host (13). Infectivity results have been expressed in two ways: (i) as tumor-initiating ability (TIA), which is the mean number of tumors induced per leaf by equal volumes of control and treated bacterial culture samples; and (ii) as per cent specific infectivity (SI), which is the mean number of tumors induced per leaf per viable bacterial cell in the inoculum, expressed relative to the control value set equal to 100%. The standard error of the mean for TIA and SI determinations are both commonly about $\pm 20\%$ of the measured value as determined in replicate tests (6, 7, 13).

RESULTS

Action of MC on the viability and TIA of *A. tumefaciens*. MC is a highly efficient bactericide for *A. tumefaciens* as shown in Table 1. Despite the rapid decline in cell viability at concentrations of 1 and 5 $\mu\text{g/ml}$, the TIA of treated cultures initially rose, then declined at a gradually increasing rate. The SI of the treated cultures increased two- to threefold at maximum, but with further time of treatment the SI returned to nearly the level of the control at about the same time the number of viable cells fell below the level necessary to observe tumor initiation. Although 1 μg of MC per ml was more effective in destroying viability relative to TIA, it was still highly effective in destroying the latter property of the bacterium, indicating that MC disrupts one of the essential components in the overall tumor induc-

TABLE 1. Effect of lethal concentrations of mitomycin C on the viability and tumor initiating ability of *Agrobacterium tumefaciens*

Mitomycin C concn	Time of exposure	Viable cells per ml ^a	Mean no. of tumors per leaf	Tumors per 10 ⁸ viable cells	Specific infectivity
$\mu\text{g/ml}$	min				%
None 1	—	6.7×10^9	50	7.5	100
	0.5	5.2×10^9	53	10	133
	1.0	2.6×10^9	61	23	307
	5.0	0.92×10^9	18	20	267
	10.0	0.34×10^9	3	8.8	117
	15.0	0.03×10^9	0	—	—
None 5	—	8.1×10^9	82	10	100
	0.5	5.7×10^9	91	16	160
	1.0	2.8×10^9	56	20	200
	5.0	0.05×10^9	0.6	12	120

^a Diluted 10^{-1} for leaf inoculations.

tion process. This is necessarily a bacterial component even though MC was present in the inoculum applied to the leaf, since as many tumors developed in the presence of MC-containing inocula after the shortest periods of treatment as were obtained in controls, and since loss of TIA is correlated with viability and time of incubation prior to inoculation.

The effect on infectivity and viability produced by exposing *A. tumefaciens* to various concentrations of MC for 10 min is shown in Fig. 1. Under these conditions, a maximal number of tumors was obtained with treatments of 5×10^{-3} μg of MC per ml. This concentration of the antibiotic had very little effect on cell viability, as subsequent experiments have borne out (G. T. Heberlein, Ph.D. Thesis, Northwestern Univ., Evanston, Ill., 1966). The SI of the treated samples increased to about twofold in this experiment at 5×10^{-3} μg of MC per ml, and then remained constant at about this level of promotion, with the possible exception of the highest concentration tested.

Since essentially maximal infectivity promotion could be obtained with 5×10^{-3} μg of MC per ml without significantly affecting cell viability,

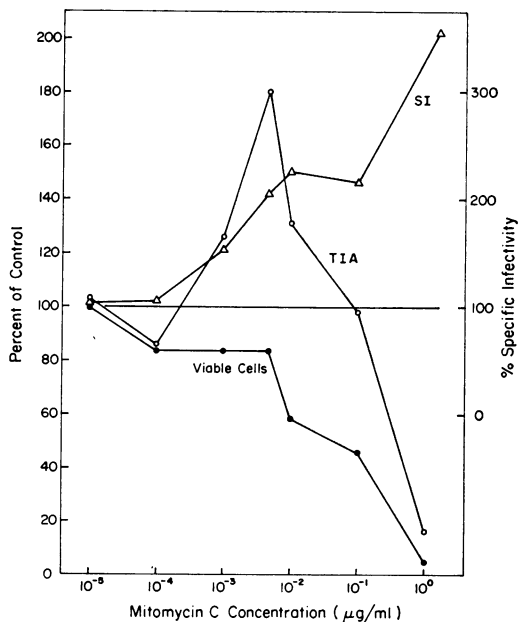


FIG. 1. Effect of 10-min treatments of *Agrobacterium tumefaciens* strain B6 with various concentrations of mitomycin C on viability (●), tumor-initiating ability (○), and per cent specific infectivity (△). The control sample contained 2.4×10^9 viable cells per ml. All samples were inoculated at a 10^{-1} dilution, the control initiating a mean of 19.7 tumors per leaf on 16 leaves.

this concentration of antibiotic was selected for further study. Typical changes in the tumor-inducing ability of a culture treated for various times with this concentration of MC are shown in Fig. 2. TIA rose to a maximum after about 10 min of treatment, but by 20 min returned to essentially the control level and remained there with longer exposures. Throughout this time, the number of viable cells remained constant. The rise in TIA is apparently due to the accumulation of MC-induced lesions, with more cells acquiring a first lesion with increasing time of treatment and more lesions occurring in cells having already received some MC-induced damage. The decrease in TIA after about 10-min treatment, however, is not readily explained in this way. Since MC lesions are repairable by bacterial DNA repair systems (1, 16, 27), this could offer a possible explanation for the decrease. There appears to be no continued accumulation of DNA lesions in these low dose experiments, since this would be accompanied by a decrease in the number of viable cells. Therefore, this dose, while sufficient to promote infectivity, is insufficient to kill even with extended treatment, allowing the possibility for repair systems to act. These results are consistent with the ultraviolet-induced infectivity promotion in indicating that a minimal amount of DNA damage must be present at the time of inoculation to obtain the effect (7). The MC promotion of infectivity is clearly a result of action on the bacterium prior to inoculation since it is only obtained after a few minutes of incubation

with the antibiotic and increases with further incubation to the maximal promotion.

Nature of the MC enhancement of TIA. As in the case of the ultraviolet-induced promotion (7), several parameters of the pinto leaf bioassay known to affect the number of tumors induced independently of the number of viable bacteria inoculated were checked to determine if they were associated with the MC infectivity promotion. No change in the rate of tumor appearance or rate of tumor growth was found in MC-treated cultures relative to controls (Heberlein, Ph.D. Thesis). MC promoted early log-phase cultures as well as older cultures, and the promotion was independent of the age of the host plant (Heberlein, Ph.D. Thesis). We conclude, as in the case of the ultraviolet results (7), that the promotion of the infectivity of *A. tumefaciens* by MC is fully comparable to the inoculation of a greater concentration of nontreated bacteria.

DISCUSSION

The characteristics of MC action in other bacterial systems and the similarities between the infectivity promotion in *A. tumefaciens* obtained with this antibiotic and with ultraviolet irradiation indicate that bacterial DNA is the primary target in this response. Since the MC promotion may be obtained under conditions where relatively little mutation might be expected, and considering the mutation frequency for individual genes, it appears impossible that this promotion is associated with a lesion at one or a few specific genetic loci. Instead, the promotion appears to stem from random lesions in the bacterial DNA, as is necessary for prophage and bacteriocin induction (15). A similar conclusion was drawn from the characteristics of the ultraviolet-induced promotion (7).

The MC promotion of *A. tumefaciens* infectivity is also similar to that observed with ultraviolet since it may be obtained under conditions resulting in considerable loss in cell viability or at doses resulting in no loss in viability. Large promotions are readily obtained under the latter circumstances with MC. This clearly demonstrates that the promotion is not due to a process resulting in a selection of highly infectious cells, but results from an actual increase in the proportion of viable cells that induce tumors. The probability with which each cell of an *A. tumefaciens* culture properly located in a susceptible wound site may induce a tumor, therefore, must initially be less than one. A random addition of some minimal number of DNA lesions is somehow capable of increasing this probability either by: (i) converting noninfectious cells to an infectious state in an

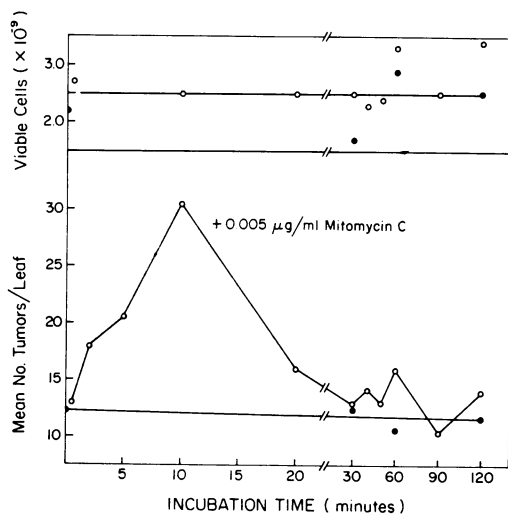


FIG. 2. Response of *Agrobacterium tumefaciens* to various periods of incubation in 0.005 μg of mitomycin C per ml: above, viability; below, tumor initiating ability. (●) Control; (○) treated samples.

all-or-none type of effect; (ii) raising the general probability of infection in all viable cells; or (iii) greatly increasing the probability of infection in one physiological class of cells normally of low specific infectivity. These possibilities cannot be distinguished at present, although it has been shown that different physiological states of *A. tumefaciens* may exhibit variations in SI of several-fold as evidenced by SI changes with culture age (13) and a decrease in SI in the absence of viability changes after certain heat treatments (14).

The induction of prophage and bacteriocins by MC occurs optimally at concentrations of about 1 $\mu\text{g/ml}$ in many of the systems that have been studied (4, 12, 17, 19, 20). This is about a 100-fold higher concentration than we found necessary for optimal infectivity promotion. Sensitivity differences to MC of this magnitude exist, however, between different species of bacteria (4, 12, 23, 26, 29), and media limiting energy metabolism may increase MC sensitivity by a factor of 10 as measured by prophage induction (19). From the rate of cell inactivation resulting from treatment of *A. tumefaciens* with 1 μg of MC per ml, it appears to be among the more sensitive species. This may be related to the high guanine-cytosine content of *A. tumefaciens* DNA (3) since the MC strand linkages appear to involve this particular base pair (10, 26).

While high concentrations of MC are necessary for optimal prophage induction, Dudnik (4) has shown that the minimal concentration of MC resulting in a 10-fold increase in the number of infectious phage particles is less than 0.01 $\mu\text{g/ml}$ for *Escherichia coli* K-12 (λ) and for *Micrococcus lysodeikticus* 53-40 (N-5). Concentrations of MC as low as 0.001 $\mu\text{g/ml}$ may also induce partial inhibition of DNA synthesis in highly sensitive organisms (23), and low concentrations have been shown to block bacterial division with the resulting formation of filamentous cells (5, 22; H. Suzuki and W. W. Kilgore, *Bacteriol. Proc.*, p. 40, 1964). Thus, several avenues appear available to account for our infectivity promotion.

Concentrations of MC of 0.5 $\mu\text{g/ml}$ or greater induce the breakdown and release into the medium of a portion of the bacterial DNA in treated cultures (22), and bacteria treated with 10 μg of MC per ml completely and irreversibly lose the ability to synthesize DNA within 10 min (18). The effectiveness of 1 and 5 μg of MC per ml in reducing the TIA of *A. tumefaciens* cultures thus indicates that the DNA of the bacterium must remain largely intact if tumor initiation is to proceed. DNA synthesis may also be required for tumor initiation, since MC may have a twofold inhibitory effect on DNA synthesis, one stemming

from the breakdown of DNA and one from the reduced ability of MC-treated DNA to serve as a primer for DNA synthesis as observed in vitro (21).

From the molecular weight of MC (28) and the conditions of our experiments, the number of MC molecules per bacterium may be estimated to be about 6,000 at a concentration of 0.01 $\mu\text{g/ml}$. The actual number of DNA cross-links induced under these conditions would undoubtedly be considerably less than this figure, however. Iyer and Szybalski (9) have estimated that a 5-min exposure to 1 μg of MC per ml results in about 50 cross-links per bacterial genome. Assuming a complete proportionality of bacterial species, exposure time, and concentration of MC, treatment with 0.01 μg of MC per ml for 10 min might be expected to result in one cross-link per *A. tumefaciens* genome. At most, therefore, only a few DNA cross-links per bacterium may be necessary for this infectivity promotion.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Fellowship 1-F1-GM-23,713-01A1 from the Division of General Medical Sciences to the senior author and by Public Health Service grant CA-05387 from the National Cancer Institute.

LITERATURE CITED

- BOYCE, R. P., AND P. HOWARD-FLANDERS. 1964. Genetic control of DNA breakdown and repair in *E. coli* K-12 treated with mitomycin C or ultraviolet light. *Z. Vererbungslehre* **95**:345-350.
- COLES, N. W., AND R. GROSS. 1965. The effect of mitomycin C on the induced synthesis of penicillinase in *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* **20**:366-371.
- DE LEY, J., M. BERNAERTS, A. RASSEL, AND J. GUILMOT. 1966. Approach to an improved taxonomy of the genus *Agrobacterium*. *J. Gen. Microbiol.* **43**:7-17.
- DUDNIK, YU. V. 1965. Induction of lysogenic culture of *Micrococcus lysodeikticus* by antibiotics selectively affecting DNA synthesis. *Federation Proc.*, Part II. **24**:T1049-1051.
- GRULA, M. M., AND E. A. GRULA. 1962. Reversal of mitomycin C-induced growth and division inhibition in a species of *Erwinia*. *Nature* **195**:1126-1127.
- HEBERLEIN, G. T., AND J. A. LIPPINCOTT. 1965. Photoreversible ultraviolet enhancement of infectivity in *Agrobacterium tumefaciens*. *J. Bacteriol.* **89**:1511-1514.
- HEBERLEIN, G. T., AND J. A. LIPPINCOTT. 1967. Ultraviolet-induced changes in the infectivity of *Agrobacterium tumefaciens*. *J. Bacteriol.* **93**:1246-1253.
- IJIMA, T., AND A. HAGEWARA. 1960. Mutagenic action of mitomycin C on *Escherichia coli*. *Nature* **185**:395-396.

9. IYER, V. N., AND W. SZYBALSKI. 1963. A molecular mechanism of mitomycin action: linking of complementary DNA strands. Proc. Natl. Acad. Sci. U.S. **50**:355-362.
10. IYER, V. N., AND W. SZYBALSKI. 1964. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. Science **145**:55-58.
11. KILGORE, W. W., AND J. GREENBERG. 1961. Filament formation and resistance to 1-methyl-3-nitro-1-nitrosoguanidine and other radiometric compounds in *Escherichia coli*. J. Bacteriol. **81**:258-266.
12. LEVINE, M. 1961. Effect of mitomycin C on interactions between temperate phages and bacteria. Virology **13**:493-499.
13. LIPPINCOTT, J. A., AND G. T. HEBERLEIN. 1965. The quantitative determination of the infectivity of *Agrobacterium tumefaciens*. Am. J. Botany **52**:856-863.
14. LIPPINCOTT, J. A., AND B. B. LIPPINCOTT. 1965. *Agrobacterium tumefaciens*: thermal inactivation of tumor-inducing ability. Science **147**:1578-1579.
15. LWOFF, A. 1953. Lysogeny. Bacteriol. Rev. **17**:269-337.
16. MAHLER, I. 1966. Effect of mitomycin C on five excision-repair mutants of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **25**:73-79.
17. MARJAI, E., AND G. IVANOVICS. 1964. The effect of different anticancer agents on inducible systems of *Bacillus megaterium*. Acta Microbiol. **11**:193-198.
18. MATSUMOTO, I., AND K. G. LARK. 1963. Altered DNA isolated from cells treated with mitomycin C. Exptl. Cell Res. **32**:192-196.
19. OTSUJI, N. 1961. The effect of glucose on the induction of Lambda phage formation by mitomycin C. Bikens J. **4**:235-241.
20. OTSUJI, N., M. SEKIGUCHI, T. IJIMA, AND Y. TAKAGI. 1959. Induction of phage formation in the lysogenic *Escherichia coli* K-12 by mitomycin C. Nature **184**:1079-1080.
21. PRICER, W. E., JR., AND A. WEISSBACH. 1964. The effect of lysogenic induction with mitomycin C on the DNA and DNA polymerase of *Escherichia coli* K-12 λ . Biochem. Biophys. Res. Commun. **14**:91-95.
22. REICH, E., A. J. SHATKIN AND E. L. TATUM. 1961. Bacteriocidal action of mitomycin C. Biochim. Biophys. Acta **53**:132-149.
23. SEKIGUCHI, M., AND Y. TAKAGI. 1960. Effect of mitomycin C on the synthesis of bacterial and viral deoxyribonucleic acid. Biochim. Biophys. Acta **41**:434-443.
24. SHIBA, S., A. TERAWAKI, T. TAGUCHI, AND J. KAWAMATA. 1958. Selective inhibition of formation of deoxyribonucleic acid in *Escherichia coli* by mitomycin C. Nature **183**:1046-1057.
25. SZYBALSKI, W. 1958. Special microbiological systems. II. Observations of chemical mutagenesis in microorganisms. Ann. N.Y. Acad. Sci. **76**:475-489.
26. SZYBALSKI, W., AND V. N. IYER. 1964. Cross-linking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics. Federation Proc. **23**:946-957.
27. TERAWAKI, A., AND J. GREENBERG. 1966. Post-treatment breakage of mitomycin C induced cross-links in deoxyribonucleic acid of *Escherichia coli*. Biochim. Biophys. Acta **119**:540-546.
28. WEBB, J. S., D. B. SOCULICH, J. H. MOWAT, J. B. PATRICK, R. W. BROSCARD, W. E. MEYER, R. P. WILLIAMS, C. F. WOLF, W. FULMOR, C. PIDACKS, AND J. E. LANCASTER. 1962. The structure of mitomycin A, B, and C and porfiromycin. J. Am. Chem. Soc. **84**:3184-3187.
29. WELKER, N. E., AND L. L. CAMPBELL. 1965. Induction and properties of a temperate bacteriophage from *Bacillus stearothermophilus*. J. Bacteriol. **89**:175-184.