DIFFERENT LETHAL EFFECTS OF MITOMYCIN C AND ACTINOMYCIN D DURING THE DIVISION CYCLE OF HELA CELLS

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ABSTRACT

The lethal actions of mitomycin C and actinomycin D were followed during the division cycle of HeLa cells. The cells were most susceptible to a 2 hr pulse of mitomycin C during the G_1 phase, whereas their sensitivity to actinomycin D was most pronounced in the S phase. Posttreatment of the cells with acetoxycycloheximide, a potent inhibitor of protein synthesis, increased the survival (colony-forming ability) of cells treated with mitomycin C but had very little effect on the survival of cells treated with actinomycin D. The significance of these findings is discussed.

INTRODUCTION

That certain chemicals have different toxicities with respect to the various stages of the cell division cycle has been observed in several types of mammalian cells in culture (1–5). Treating synchronous cells with specifically acting chemicals and observing fluctuations in their survival may be a possible means of gaining insight into alterations in vital cell constituents during the cell cycle.

In the present investigation, we studied the lethal effects of mitomycin C and actinomycin D on synchronous HeLa cells in terms of cell colony formation. Both of these antibiotics are known to affect DNA, but each in a different manner. We also studied the effect of acetoxycycloheximide, an inhibitor of protein synthesis, on these treated cells. Acetoxycycloheximide was found to reverse, to some extent, the lethal effects of mitomycin C, much in the fashion of the restorative effect it has on X-irradiated cells.[1] Similarly, excess thymidine at a concentration which inhibits DNA synthesis (6, 7) had a restorative effect on mitomycin C–treated cells.

MATERIALS AND METHODS

The HeLa S-3 cells used in this study were maintained in Eagle's medium supplemented with 0.1 mM nonessential amino acids and 10% calf serum. Culture conditions, scoring survival by colony formation, maintenance, and media composition have been described elsewhere (7, 8).[1] Synchronous cultures were initiated by plating mitotic cells which had been selectively removed by controlled agitation from a monolayer of cells in Ca^{2+}-deficient medium (8). Mitomycin C or actinomycin D was added to fresh

[1] Djordjevic, B., J. H. Kim, and S. H. Kim. 1968. Modification of radiation response in synchronized HeLa cells by metabolic inhibitors. In preparation.

medium shortly before each experiment, and this supplemented medium was added to emptied dishes at specified times. Acetoxycycloheximide (ACH) and thymidine (TdR) were added directly to medium from a 50× stock solution to yield the desired final concentrations. The drugs were added after the cells had become attached; a 3 hr period after plating was allowed for cell attachment.

Incorporation of tritiated precursors for estimation of DNA, RNA, and protein synthesis was studied in 5–10 × 10^4 randomly dividing cells in the absence or presence of mitomycin C for certain periods of time. The cells were harvested by trypsinization and filtered on millipore filters as outlined previously (5). The filter was suspended in 5 ml of toluene containing 4 g/liter 2,5-diphenyloxazole (PPO) and 50 mg/liter 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) and counted in a liquid scintillation counter.

RESULTS

Effects of Drug Concentration and Duration of Treatment on Colony Formation in Randomly Dividing Cells

To determine the dose response of HeLa cells to mitomycin C and actinomycin D, randomly dividing cells were exposed to several different concentrations of each antibiotic for various periods of time. Typical dose-survival curves were obtained, as shown in Figs. 1 and 2. Mitomycin C displays a disproportionally small effect at short exposure times as compared to longer times; that is, a shoulder region is evident in the survival curve (Fig. 1). Actinomycin D reduces cell viability (colony-forming ability) exponentially, since there is no apparent shoulder in the curve when survival is plotted semilogarithmically as a function of exposure to this compound (Fig. 2). On the basis of these results, for further experiments a time of exposure to the two antibiotics was chosen which is long enough to encompass the shoulder region of the curve (in the case of mitomycin C), yet short enough to permit a reasonable correlation of drug action with particular metabolic landmarks in the division cycle. Accordingly, a 2-hr incubation with these antibiotics was chosen for the treatment of synchronous cells.

Effect of Mitomycin C on Synthesis of DNA, RNA, and Protein

A series of 18-hr random cultures of HeLa cells was exposed to 0.1 μg/ml mitomycin C

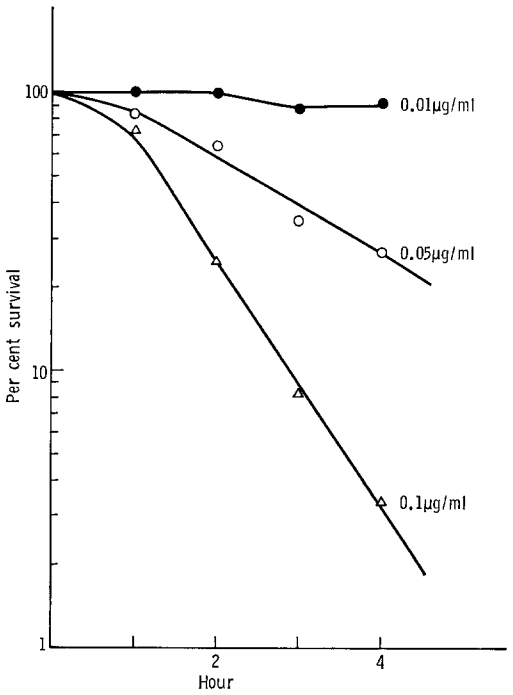

FIGURE 1 Survival (colony-forming ability) in randomly dividing HeLa cells exposed to different concentrations of mitomycin C for different periods of time. Drug was added to cells 18 hr after plating.

together with thymidine-^{3}H, uridine-^{3}H, or valine-^{3}H for different periods of time. A control series was similarly set up but without the antibiotic. It is evident from the results shown in Table I that macromolecular synthesis of DNA, RNA, and protein is relatively insensitive to the drug during the first few hours of treatment. This result is in agreement with findings on other cultured mammalian cells treated with mitomycin C (9).

Fluctuations of Lethal Effects of Mitomycin C and Actinomycin D During Cell Division Cycle; Effect of Acetoxycycloheximide in Modifying Toxicity of these Antibiotics

Fig. 3 shows the survival of synchronous HeLa cells exposed for 2 hr to mitomycin C or actinomycin D during the division cycle. The two drugs differ considerably in toxicity pattern (in terms of inactivation of colony-forming ability); mitomycin C is maximally effective in killing HeLa cells when they are in the G_1 phase, whereas

actinomycin D is most toxic to the cells in the early part of their synthetic (S) phase.

In a further effort to characterize the cyclic response of cells to the lethal actions of mitomycin C and actinomycin D, G_2-phase cells were selected by means of the combined action of thymidine-^{3}H and vinblastine (5, 10, 11), and the effects on these cells of mitomycin C and actinomycin D were examined. The method consists of treating cells with thymidine-^{3}H of high specific activity (2 μc/ml medium, 14 c/mmole), which selectively kills S-phase cells during a 1 hr treatment, and with vinblastine sulfate (0.03 μg/ml) which does not interfere with cell progression through the interphase but arrests cells in metaphase when they are easily removed from the rest of the population. S-phase cells were also selected by treating synchronous cultures with a combination of a high concentration of nonradioactive thymidine and vinblastine, as described earlier (5). Table II shows that, relative to the response shown by S-phase cells, the sensitivity of G_2-phase cells to mitomycin C increases, but their sensitivity to actinomycin D decreases although the differences are not large.

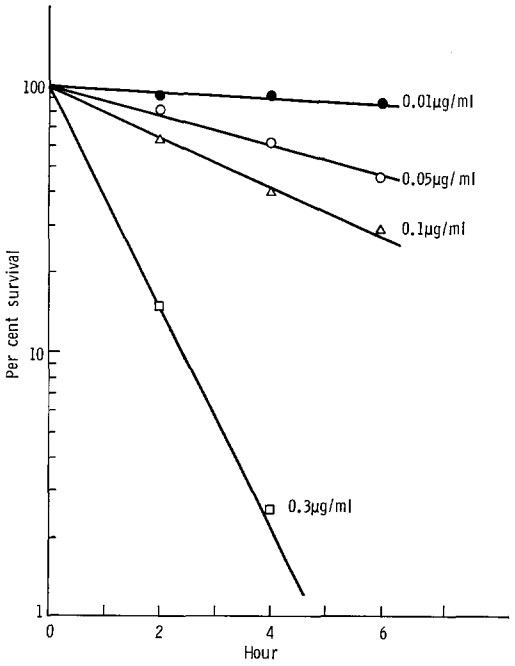

FIGURE 2 Survival in randomly dividing HeLa cells exposed to various concentrations of actinomycin D for different periods of time. Drug was added to cells 18 hr after plating.

The restorative effect of acetoxycycloheximide (ACH) when added to cells after treatment with mitomycin C is shown in Fig. 4. ACH strongly inhibits synthesis of protein and, to a lesser extent, of DNA, but it has little effect on RNA synthesis.[1] As can be seen from Fig. 4, ACH restores viability to mitomycin-treated cells; it has very little effect on actinomycin-treated cells (data not shown).

TABLE I

Incorporation of Labeled Precursors into DNA, RNA, and Protein of HeLa Cells in the Presence of Mitomycin C

Incubation time	Thymidine-^{3}H		Uridie-^{3}H		Valine-^{3}H	
	Control	Mitomycin treated	Control	Mitomycin treated	Control	Mitomycin treated
hr						
1.0	4,967*	5,292	6,016	7,916	285	311
2.0	11,410	10,576	12,095	14,163	693	552
3.0	15,675	15,267	27,087	24,801	997	959
4.0	18,758	16,515	40,690	31,617	1,320	1,388

Labeled precursors (thymidine-^{3}H: 0.5 μc/ml, 13.0 c/mmole; uridine-^{3}H: 1 μc/ml, 9.0 c/mmole; valine-^{3}H: 1 μc/ml, 4.0 c/mmole) were added to about 5×10^4 randomly dividing cells 18 hr after plating with or without 0.1 μg/ml mitomycin C. At time intervals, cells were harvested by trypsinization, and their radioactivity was determined as outlined in Materials and Methods.

* Figures are counts per minute per plate.

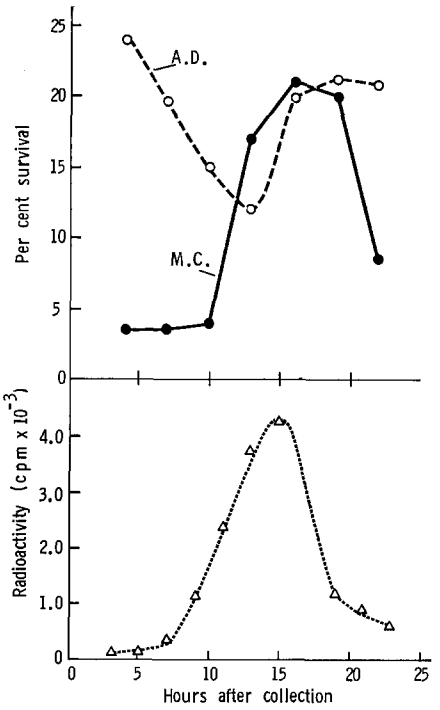

FIGURE 3 Survival of synchronously growing HeLa cells exposed to 0.1 μg/ml of mitomycin C (*M.C.*) or 0.3 μg/ml of actinomycin D (*A.D.*) for 2 hr during different parts of the division cycle (upper graph). Lower graph: DNA synthesis in the same culture as measured by the incorporation of thymidine-^{3}H during 30-min pulses at different times during the cycle.

Modification of the Lethal Effect of Mitomycin C by Pretreatment of Synchronized Cells with Metabolic Inhibitors

It was of interest to determine whether ACH treatment given before treatment with mitomycin C has a modifying effect on cell survival similar to that produced when ACH is given after treatment with mitomycin C. At 8 hr after the initiation of the culture, synchronous cells were treated for 2 hr with mitomycin C. ACH, and/or 2×10^{-3} M thymidine, also active in restoring irradiated cells,[1] were included in the 3rd–8th hr when pretreatment effects were being tested and in the 10th–13th hr when posttreatment effects were being examined.

Table III shows that the pretreatment of cells with ACH has little effect on mitomycin C–caused lethality compared with its effect following mitomycin C treatment. Such a finding is in accordance with the view that posttreatment with ACH favors repair.

DISCUSSION

That there are differences in the lethal actions of mitomycin C and actinomycin D during the division cycle of HeLa cells is not surprising in view of the different modes of action of the two antibiotics. Whereas the toxicity of mitomycin C is greatest during the early phases (G_1) of the cycle, that of actinomycin D is highest in the early part of the S phase. The fact that both mitomycin C and actinomycin D form complexes with native DNA may account for their toxic actions. Although mitomycin C will bind to other cell constituents (12), its lethal action, at least in bacteria, is thought to result from its ability to cross-link complementary DNA strands (13). Actinomycin D binds specifically to DNA, owing to the unique stereochemical properties of the antibiotic and the macromolecule (14, 15).

The observed toxicity variations during the cycle might be explained in several ways: *a*, cell permeability to drugs may vary; *b*, accessibility of DNA in chromosomal structures may vary during the cycle, e.g. receptive sites on the macro-

TABLE II

Lethal Effects of Mitomycin C and Actinomycin D on "Purified" S-phase and G_2-phase Cells

Supplements	Survival of corresponding controls	
	Mitomycin C (20–22 hr)	Actinomycin D (20–22 hr)
	%	%
No supplement	19.4	57
VLB 3–20 hr	33.0	61.8
TDR 16–20 hr		
VLB 3–20 hr	20.6	71.5
TDR-^{3}H 19–20 hr		

G_2 cells were selected by treating mitotically synchronized cells with a combination of vinblastine sulfate (VLB, 0.03 μg/ml) and tritiated thymidine of high specific activity (TDR-^{3}H, 2 μc/ml, 14 c/mmole) as described previously (11). S-phase cells were selected by treating synchronous cells with a combination of VLB and excess thymidine (TDR, 500 μg/ml). Hours indicate time elapsed between the initiation of synchronous culture and the particular treatment performed. Concentration of drugs: mitomycin C: 0.1 μg/ml; actinomycin D: 0.1 μg/ml.

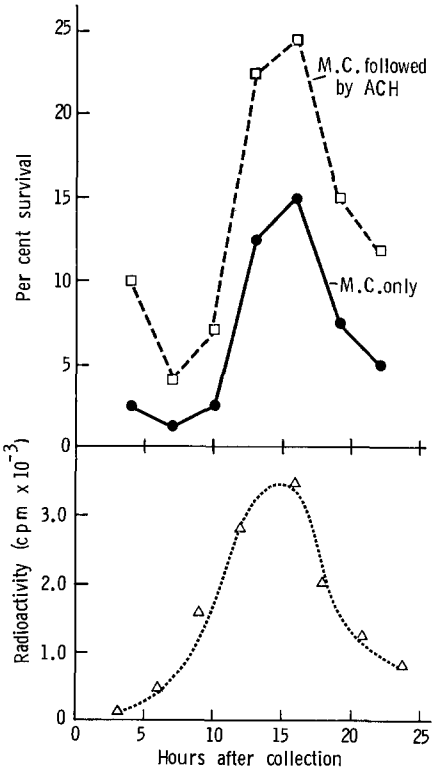

FIGURE 4 Survival of synchronous cells after 2-hr pulse treatment with 0.1 μg/ml of mitomycin C (*M.C.*) during different parts of the division cycle, with or without subsequent 3-hour treatment with 5 μg/ml of acetoxycycloheximide (upper graph). DNA synthesis in the same culture is shown in the lower graph.

molecule may be changed by contraction of chromosomes or masked by products of its template activity; *c*, intrinsic sensitivity of the DNA molecule may itself vary; and *d*, posttreatment repair processes may be acting in a different manner during the cycle. Possibility *a*, that changes in permeability may be so finely regulated as to govern the precise amount of antibiotic delivered to the sensitive sites in the cell, seems unlikely. Possibility *d* may have some importance, but most probably in conjunction with processes outlined in possibilities *b* and *c*, as will be discussed below.

It is tempting to speculate that the degree of damage caused by some toxic chemicals depends on the physical state of DNA. It has been shown (16) that the physical state of DNA undergoes changes in vivo, i.e. that the macromolecule is transiently and partially denatured in preparation for replication. In the case of mitomycin C, it has been postulated that the biological effect of bifunctional alkylating agents (e.g. nitrogen mustard, mitomycin C) is due to the formation of interstrand cross-links, i.e. linkage across complementary strands in DNA. It may be speculated that mitomycin C cross-links DNA to a greater extent earlier in G_1, when the strands of the macromolecule are in a more favorable juxtaposition. With actinomycin D, the foregoing considerations may not apply; instead, processes participating in repair may be critical in the toxicity of this antibiotic. Such a situation could exist when partially separated strands (e.g. during S) are inactivated and thus interfere with the capacity of both the damaged and the undamaged strands to copy from each other. On the other hand, while there seems to be good correlation between cross-linking and the lethal action of mitomycin C in bacteria (13), very little cross-linking was found in DNA isolated from inactivated D98S cells (17). Moreover, much of the lethal effect could be reversed by parallel or even delayed treatment with $NaBH_4$ (18). These observations, together with our finding that the toxic action of mitomycin C may be somewhat reversed by posttreatment with ACH, argue against the effect of cross-linking, thought to be an irreparable process (19), as the sole cause of lethal action.

TABLE III

Effect of Combined Treatment by Mitomycin C and Acetoxycycloheximide or Excess Thymidine on Survival of HeLa Cells

Compounds	Survival of cells treated by mitomycin C*	
	Pretreatment‡	Posttreatment§
	%	%
None	2.8	2.8
ACH	4.4	13.9
TDR	3.7	9.4

* Cells were incubated with 0.1 μg/ml of mitomycin C 8–10 hr after plating the mitotically synchronized cells. Corresponding controls with additional treatment (ACH or TDR only) were taken as 100%.
‡ Pretreatment consisted of incubating cells 3–8 hr with acetoxycycloheximide (ACH, 5 μg/ml) or thymidine (TDR, 500 μg/ml).
§ Posttreatment consisted of incubating cells 10–13 hr after initiating synchronous cultures with the above concentrations of ACH or TDR.

At the relatively low concentration of mitomycin C used in this study, the lethal effects may be also exerted by alkylation of membranes and other cytoplasmic components (17). Such processes may require more time to be effective and are reflected in the sigmoidal shape of the survival curve of cells exposed for various periods of time to mitomycin C. Both modes of lethal action may actually operate in mitomycin C–treated cells, and it is therefore possible that the periodic fluctuations in lethal action during the cycle are due to cross-linking of DNA strands, while the reparable portion is due to other effects of mitomycin C.

Again, it is hoped that further experimentation will clarify the processes leading to cell death after treatment with mitomycin C and actinomycin D.

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