MITOMYCIN C INDUCED INCREASES IN THE ACTIVITIES OF THE DEOXYRIBONUCLEASES OF HELA CELLS

George P. Studzinski and Larry S. Cohen

Department of Pathology, Jefferson Medical College, Philadelphia, Pa.

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Mitomycin C (MC) selectively inhibits DNA synthesis in bacterial (Shiba et al., 1959) and mammalian cells (Reich and Franklin, 1961) allowing RNA and protein synthesis to continue for a considerable time, but arresting cell division. Recent studies on the mode of its action have shown that in bacteria MC cross-links complementary strands of DNA, and thus prevents the unwinding of the DNA helix necessary for replication (Iyer and Szybalski, 1963; Matsumoto and Lark, 1963). On the other hand, the finding that MC action is often accompanied by the accumulation of acid soluble deoxyribose compounds in the cell has led to the view that the primary action of MC is to depolymerize DNA (Reich et al., 1960), and Kersten (1962) suggested that this is caused by an activation of a cellular DNase. Increased activity of a DNase has been found following treatment of E. coli with MC (Nakata et al., 1961; Kersten et al., 1966).

This communication shows that treatment with MC results in increased cell content of two of the DNases present in HeLa cells. Nevertheless, the DNA content of the inhibited cells is not lowered and acid soluble deoxyribose compounds do not accumulate to any extent. It is therefore doubtful whether the elevated DNase activity plays a part in the mechanism of the inhibitory action of MC on HeLa cells.

Materials and Methods: The cells were derived from the clonal line HeLa (-af) described by Saksela et al. (1961). The cells were grown in petri dishes as described previously (Studzinski and Love, 1964). They were harvested by scraping into the medium, counted after dilution of a small sample with versene in a Coulter particle counter, and the centrifuged cell pellet was washed with isotonic saline. The cells were resuspended in distilled water ($20x10^6$ cells per ml), disrupted by sonication for 20 minutes in a Raytheon Sonic Oscillator, and the crude sonicate was used for enzyme assays. Protein was measured by the method of Lowry et al. (1951), DNA by Burton's (1956) diphenylamine reaction after separation of the nucleic acids by the Schmidt-Thannhauser procedure (Fleck and Munro, 1962). MC and puromycin were obtained from N. B. Co., Cleveland, Ohio, and actinomycin D was a gift from Merck Sharp and Dohme, Rahway, N. J.

Results: Two of the several DNases mentioned by Eron and McAuslan (1966) can be easily demonstrated in the HeLa cells by the methods employed here. One has a slightly alkaline pH optimum, requires Mg ions, and is active only against heat denatured, presumably single-stranded, DNA. The other has an acid pH optimum, is active against both native and denatured DNA, and is present in the lysosomes of HeLa cells (Studzinski and Cohen, in preparation). Fig. 1 shows that both activities increase in cells treated with MC. At early times of treatment the increase is rather more pronounced for alkaline DNase; but, after 48 hours of MC action, the levels of activity of both enzymes approach 300% of the control values.

The concentration of MC employed, though it inhibits cell division, allows some protein synthesis in HeLa cells (Kuroda and Furuyama, 1963). This suggested that the increased per cell content of the DNases may be simply

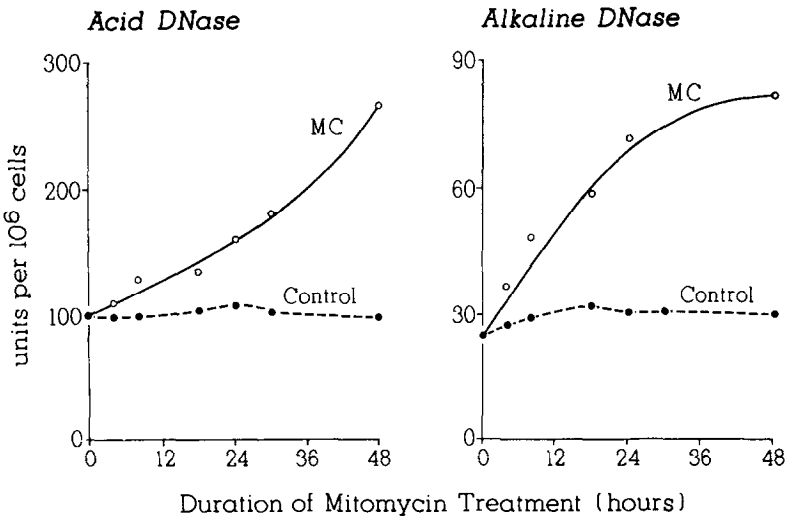

Fig. 1. Increased DNase activities of HeLa cells after varying periods of treatment with 0.1 μg/ml of mitomycin C. Alkaline DNase was measured in triplicates by incubating 0.2 ml sonicate with 100 mg of heat-denatured DNA and 0.9 mg of $MgSO_4$ in a total volume of 1.5 ml of veronal-acetate buffer pH 8.5. The assay conditions for acid DNase differed in that native DNA was used, $MgSO_4$ was omitted, the amount of sonicate was 0.1 ml and the pH was 5.0. Incubation at 37°C for 60 min. was terminated by addition of 0.8 ml of 0.6 N PCA and transfer to an ice bath. The mixture was centrifuged at 1000xg for 20 min. and the deoxyribose content of the supernatent was measured by the method of Burton (1956). One unit of enzyme activity is taken as the amount which produces 0.1 μg of acid soluble deoxyribose in 60 min. under the conditions described above.

due to continuation of the normal rate of production of these enzymes in non-dividing cells. The data in Table I show, however, that the content of both DNases in treated cultures as a whole considerably exceeds the content of control cultures. These increases are not due to an abnormal retention of the enzymes by treated cells, since DNases are not released from HeLa cells and could not be detected in the incubation medium of control or treated cultures.

In spite of the elevated activities of both DNases, MC-treated HeLa cells have normal content of DNA (Table II). In addition, measurement of acid-soluble deoxyribose compounds by Burton's (1956) method confirmed the observation of

Table I. Comparison of the Increases in Cell Number, Protein and DNase Content of Control and Mitomycin-Treated Cultures.

	Cell number per culture $x10^{-6}$	Protein content mg/culture	Acid DNase units/culture	Alkaline DNase units/culture
0 hrs.	4.5	1.41	444	159
48 hrs. control	17.8	3.62	1530	615
48 hrs. treated	4.8	3.28	2195	910

Mean values of 3 experiments. MC was 0.1 μg/ml.

Magee and Miller (1962) that there was no extensive breakdown of DNA in MC-treated HeLa cells.

Table II. DNA Content of HeLa Cells Treated with 0.1 μg/ml of MC.

Hrs. of treatment	0	24	48	72
Control [+]	14.7	15.2	13.9	13.6
MC-treated [+]	----	15.1	14.2	14.1

[+] μg DNA per 10^6 cells. Mean values of 3 experiments.

The observed elevations in enzyme activities can be due to either synthesis of new enzyme molecules or to activation of the existing enzymes, e.g. by removal of an inhibitor. MC did not activate the enzymes when added to the assay mixture. Fig. 2. shows the effect of 8 hours of simultaneous treatment with puromycin (in concentration which effectively inhibits protein synthesis (Mueller et al., 1962)) and MC on cells which were pretreated with MC for 20 hours. Both acid and alkaline DNase activities are severely depressed by puromycin in MC-treated cells. The results suggest that the elevation of both enzyme activities require protein synthesis. The point was further studied

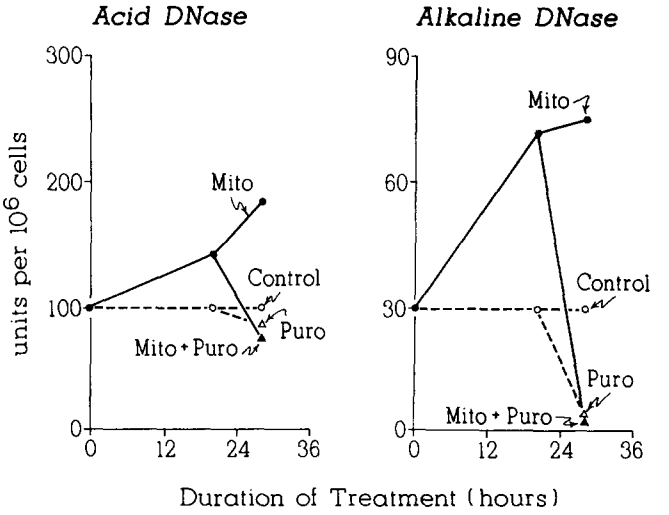

Fig. 2. Effect of puromycin (100 μg/ml) on DNase activities of control and mitomycin-pretreated cells.

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in experiments of 18 hours duration in which no pretreatment was given (Table III). Actinomycin was also used, in a concentration known to inhibit effectively DNA-primed RNA synthesis (e.g. Littlefield, 1966), in an effort to determine if the stimulus for the formation of new DNase molecules is mediated by RNA "messages" from the genome. The results shown in Table III indicate that in the presence of either puromycin or actinomycin D, MC does not cause increased activities of the DNases. Furthermore, it should be noted that increased activities of the DNases are not seen after treatment of the cells with puromycin or actinomycin D.

Discussion: Since no evidence of enhanced degradation of DNA was found, the elevated DNase activities are more likely to be secondary consequences of MC action rather than the primary cause of inhibition of HeLa cell replication. The exact stimulus for the elevation remains a matter for speculation, but the experiments with puromycin and actinomycin D suggest that new enzyme

Table III. Effects of Puromycin and Actinomycin D on DNase Activities of Control and Mitomycin-Treated Cells. 18 hrs. treatment with: MC 0.1 μg/ml, puromycin 100 μg/ml, actinomycin D 1 μg/ml.

	Acid DNase		Alkaline DNase	
	Units per 10^6 cells	Units per mg protein	Units per 10^6 cells	Units per mg protein
Controls	94.2	435	37.4	167
MC	160.2 (170)	553 (127)	71.8 (192)	248 (149)
MC + Puromycin	76.3 (81)	468 (108)	4.0 (11)	19 (11)
Puromycin	78.4 (83)	381 (88)	4.8 (13)	24 (14)
MC+Actinomycin D	92.5 (98)	463 (107)	6.8 (18)	22 (13)
Actinomycin D	88.2 (94)	413 (95)	4.4 (12)	26 (16)

Means of 3 experiments. The figures in parentheses are percentages of control value.

molecules are formed, and that this process is under the direction of the DNA code.

The ability of HeLa cells to continue to synthesize RNA and protein when DNA synthesis and cell division are inhibited may be considered as "unbalanced growth". The increased production of the enzymes described here shows that at least some of the proteins produced under these conditions are normal in that they are capable of functioning as enzymes. This demonstrates functional integrity of at least a part of the genome of cells with DNA which cannot replicate.

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