Reductive Activation and Inactivation of Mitomycin As Studied with Human and Bacterial Cell Cultures

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SUMMARY

Sodium borohydride at noninhibitory concentrations (50 μg/ml) reverses almost completely the lethal effects of mitomycin C when added within 5 min (human cell cultures) or 10 sec (bacteria) after cell exposure to the antibiotic. Sodium cyanide, dinitrophenol, and phenethyl alcohol, shown previously to augment the bactericidal effects of mitomycin, have no influence on the lethal action of this antibiotic for human cells.

Mitomycin is nonreactive in its natural oxidized (quinone) form, but becomes converted to a reactive "alkylating" intermediate when reduced either chemically or enzymically (1, 2). The cytocidal effects of mitomycin are the result of crosslinking of the complementary DNA strands by this intracellularly reduced intermediate (2). Analogous *in vitro* crosslinking of purified DNA can be achieved by reduction of mitomycin in the presence of DNA, using sodium hydrosulfite ($Na_2S_2O_4$) or sodium borohydride ($NaBH_4$) (1, 2). In the present study an attempt was made to evaluate the combined action of mitomycin and chemical reducing agents on human and bacterial cells in the hope that the results might be of some value in designing chemotherapeutic strategies for either selective activation or selective inactivation of this antineoplastic antibiotic.

To determine cell survival, the D98S human cell line was exposed to mitomycin C (MC) and $NaBH_4$ under the conditions outlined in the legend to Fig. 1. $NaBH_4$ was selected as the reducing agent, since it did not exhibit any toxicity toward D98S cells at concentrations up to 100 μg/ml. Another reducing agent, $Na_2S_2O_4$, was toxic at concentrations above 0.5 μg/ml (90, 47, and 1.5% survival at 0.5, 5, and 10 μg/ml, respectively; 2 hr exposure). Figure 1 indicates that under *in vivo* conditions $NaBH_4$, instead of enhancing MC action, abolished its cytotoxic effects, either completely (up to 0.1 μg MC/ml) or very substantially (at 0.5 and 5 μg MC/ml). Moreover, it appears that MC exerts its irreversibly lethal effects rather slowly, since MC-exposed cells could still be resuscitated by prompt (Fig. 1, curve *C*) or even somewhat delayed (Fig. 1, curves *D* and *E*) reduction with $NaBH_4$. Although it was known that enzymic or chemical reduction of MC leads to rapid loss of its activity (1–4), the *in vivo* effect of the reducing agent and MC combination could not have been easily predicted, since reduction of MC was found to be obligatory for its *in vitro* reaction with nucleic acids (1, 2). The same experiments indicate that MC is slow in reaching and irreversibly inactivating these critical structures (DNA?), and that the enzymic reductive system of the cell is fully capable of "activating" MC without any synergistic help from extracellular reducing agents. The DNA of MC-killed human cells is crosslinked, as shown in earlier studies (1, 5).

When $NaBH_4$ was added 5 min prior to

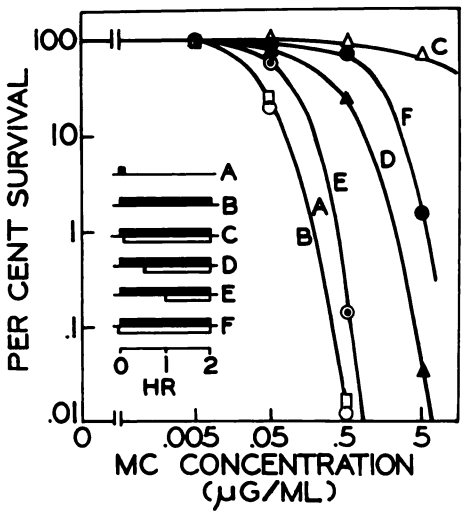

FIG. 1. *Inactivation of D98S cells by mitomycin C (MC) in the absence (curves A,B) or presence (curves C–F) of $NaBH_4$*

Curves represent the survival of D98S cells scored as the percentage of cells giving rise to clones. Plastic dishes (60 mm diameter) were seeded with 5000–10,000 D98S cells, incubated overnight in E_{90} medium to permit firm cell attachment, and then supplemented with the indicated concentrations of MC and 50 μg $NaBH_4$/ml. The schedule of cell exposure to MC (solid horizontal bars) and to $NaBH_4$ (open horizontal bars) is represented by the bar diagram on the left side of the figure: *A*, 5 min exposure to MC; *B*, 2 hr exposure to MC; *C*, exposure to MC (0 to 120 min) and to $NaBH_4$ (5 to 120 min); *D*, exposure to MC (0 to 120 min) and to $NaBH_4$ (30 to 120 min); *E*, exposure to MC (0 to 120 min) and to $NaBH_4$ (60 to 120 min); *F*, exposure to MC (0 to 120 min) and to $NaBH_4$ (−5 to 120 min). The treatment was terminated by total replacement of the medium with 5 ml of fresh E_{90} medium. The colonies (clones) formed by the surviving cells were stained and counted after 7–9 days of incubation. The methods of cell cultivation, plating, incubation (37° in 5% CO_2 atmosphere) and scoring were described earlier (3, 7) as was E_{90} medium, consisting of Eagle's medium supplemented with 10% horse serum.

addition of MC (curve *F*), cell survival was lower than in experiment *C*. The explanation of this result, which at first appeared paradoxical, lies in the rapid breakdown of $NaBH_4$ in E_{90} medium, as determined by a variety of experiments. When schedule *F* was amended by a second introduction of $NaBH_4$ (50 μg/ml) within 5 min after MC addition, survival similar to or higher than that represented by curve *C* was obtained. When the time interval (ΔT) between the $NaBH_4$ (50 μg/ml) and MC additions was varied, the slope of the survival curves covered a whole spectrum between curves *A* (ΔT more than 15 min) and *C* (ΔT less than 10 sec).

To determine whether MC is irreversibly bound by the cell or could be removed by extensive washing (Table 1), the cells were treated in a manner similar to that used in schedules *A* (experiments I and II) and *C* (cf. Fig. 1), with the latter total treatment period reduced to 10 min (experiments III and IV) and with an additional variant (experiments II and IV) consisting of two 5-ml rinses with balanced salt solution after removal of the MC-containing medium. Two rinses were about as effective in reversing the toxic effects of MC as addition of $NaBH_4$. Comparison, however, of experiments II and IV (0.5–25 μg MC/ml) shows clearly that $NaBH_4$ has a resuscitating effect beyond that of cell washing alone.

In a bacterial system it was found that MC lethality could also be counteracted by $NaBH_4$, but only when the latter was added very soon after MC. Thus survival of *Escherichia coli* B (1.3% after 10 min exposure to 2 μg MC/ml minimal medium) could be increased to 70% or to 8% when $NaBH_4$ (50 μg/ml) was added at 10 sec or 5 min after MC exposure, respectively. Inactivation of the bacteria was even more enhanced when E_{90} medium (free of penicillin and streptomycin) was used, confirming that MC killing of bacteria is more rapid than MC killing of mammalian cells. The kinetics of inactivation of D98S cells in balanced salt solution were approximately the same as shown in Fig. 1,*A–E*.

When these studies were almost completed, it was reported that the lethal effects of MC for bacteria could be augmented by simultaneous exposure to subtoxic concentrations of phenethyl alcohol (PEA), dinitrophenol (DNP), or sodium cyanide (NaCN) (6). These results were confirmed in this laboratory, using *E. coli*

TABLE 1

Resuscitation of MC-treated D98S cells by $NaBH_4$ and by two cell washings

The cells were treated either as outlined in the legend to Fig. 1 (experiments I and III) or with two additional rinses with saline A(3) after the removal of the medium and the agents (experiments II and IV).

Expt. no.	MC exposure (min)	$NaBH_4$ exposure (min)	Cell washings	MC concentration (μg/ml)				
				0	0.05	0.5	5	25
				Survival (%)				
I	0–5	None	0	100	19	<0.02	<0.02	<0.02
II	0–5	None	2×	100	94	70	0.07	<0.02
III	0–10	5–10	0	100	90	60	<0.02	<0.02
IV	0–10	5–10	2×	100	100	98	86	1

B. However, all three agents employed at subinhibitory concentrations (5 μg PEA/ml, 50 μg DNP/ml, or 50 μg NaCN/ml) were without any effect on the killing of D98S cells by MC in concentrations between 0.005 and 5 μg/ml. More detailed study of this discrepancy and its relationship to MC activation and various DNA repair mechanisms is in progress.

It could be concluded that neither of the approaches explored in the present study led to the potentiation of the cytotoxic effects of MC as directed toward human cell cultures. On the contrary, reducing agents, when applied early enough, could be considered as antidotes to the toxic effects of MC.

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