Communications to the Editor

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SYNERGISTIC EFFECT OF GLYCEROL ON CYTOTOXICITY OF BLEOMYCIN IN CULTURED CHINESE HAMSTER V79 CELLS

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The cytotoxicity of bleomycin (BLM) in cultured Chinese hamster V79 cells was greatly potentiated by the simultaneous treatment with 10% glycerol. The treatment with 1 μg/ml BLM for 1 h did not affect the survival fraction, whereas the same treatment in 10% glycerol reduced it to 10^{-3}. No combinations of glycerol with adriamycin, paraquat, hydrogen peroxide, or N-methyl-N-nitrosourea potentiated the cytotoxicities of these chemicals.

KEYWORDS —— bleomycin; Chinese hamster V79; glycerol; cytotoxicity

The cytotoxicity of bleomycin (BLM), an active oxygen-mediated anticancer agent, is potentiated in combination with hyperthermia above 42°C,[1-3] although the mechanism involved is not clear. On the other hand, glycerol protects cells against heat-killing.[4,5] The mechanism is considered to involve the stabilizing effect of glycerol on intracellular and/or membrane proteins and its regulating effect on the membrane lipid fluidity against hyperthermia.[6,7] One might, therefore, expect that the hyperthermic synergism in the cytotoxicity of BLM would be reduced by the presence of glycerol, since glycerol protects cells against hyperthermic damage. As a matter of fact, erythritol or galactose, which are known as heat-protective polyols, reduces the synergistic interaction of BLM and hyperthermia[1] The combination with glycerol reduces the toxicity of vincristin at 37°C, possibly through stabilization of the cell microtubules.[7] In the present study, contrary to our expectation, heat-protective glycerol dramatically potentiated the cytotoxicity of BLM, whether hyperthermia was present or not. This paper describes the potentiation of BLM-cytotoxicity in cultured Chinese hamster V79 cells caused by simultaneous and sequential combinations with glycerol. Combinations of some other bioactive compounds with glycerol were also examined, including adriamycin, paraquat, and hydrogen peroxide, which are active oxygen-mediated toxicants, and N-methyl-N-nitrosourea (MNU), a genotoxic alkylating agent.

EXPERIMENTAL

V79 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum (GIBCO) at 37°C in a humidified incubator with 5% CO_2 in air. BLM was purchased from Nippon Kayaku Co., Ltd. (Tokyo), adriamycin (doxorubicin hydrochloride) from Kyowa Hakkou Ind., Ltd. (Tokyo), paraquat from Wakko Pure Chem.

Ind., Ltd. (Tokyo). MNU was synthesized in our laboratory.

The cells in an early log-phase were treated with EDTA-trypsin and collected by centrifugation. The prepared cells were suspended in the same medium at a rate of 5×10^6 cells/ml. This suspension (0.2 ml) was added to 0.8 ml of Dulbecco's PBS containing appropriate concentrations of a chemical and/or glycerol and incubated for 1 h with gentle shaking. The cells were then collected by centrifugation and washed with the PBS. In the sequential treatments, the subsequent treatment was done in the same way immediately after the first treatment. The cells were resuspended in the culture medium and diluted with PBS to an appropriate volume so that the surviving cell density became 1000-2000 cells/ml. The cell suspension (0.1 ml) was then seeded in 4 ml of culture medium in a 60-mm culture dish. After 7-10 days' incubation, the colonies formed were fixed, stained, and counted.

RESULTS

No appreciable cytotoxicity was shown in the cells treated with less than 10 v/v% glycerol alone in PBS containing 20 v/v% culture medium for 1 h. As previously documented, 10 v/v% glycerol protected cells against hyperthermia (43°C, 1 h). Some experimental data are shown in the following table. (The cell numbers are the average of four experiments).

Treatment (°C)	No. of Surviving cells	Ratio
37 (1h) without glycerol	4.50×10^5	(1.0)
37 (1h) with glycerol	5.62×10^5	1.2
43 (1h) without glycerol	3.79×10^4	0.084
43 (1h) with glycerol	1.95×10^5	0.43

Synergistic Cytotoxicity of BLM and Glycerol in a Simultaneous Treatment at 37°C

The cell-killing by simultaneous treatment with BLM and glycerol (10%) is extraordinarily potentiated at 37°C over that induced by BLM alone, as shown in Fig. 1A. As shown in Fig. 1B, the synergistic effect of glycerol is sharply dependent on the concentration of glycerol.

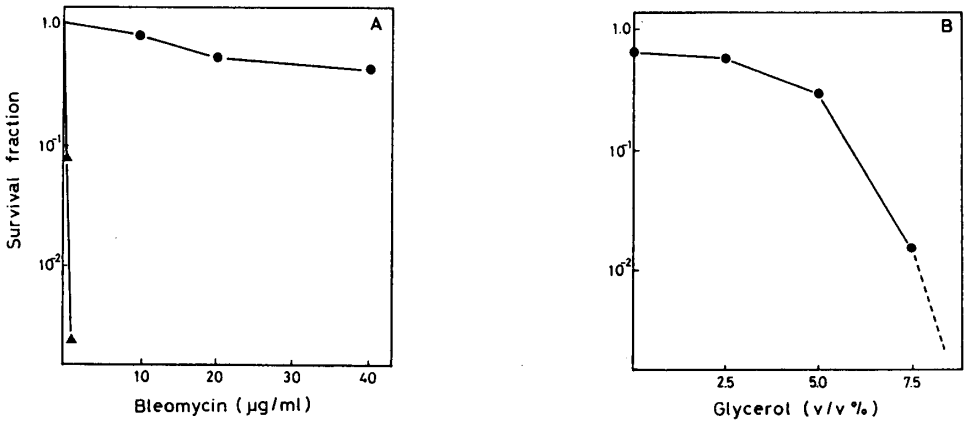

Fig. 1. (**A**) Enhancement Effect of Glycerol (10%) on BLM Cytotoxicity after 1 h Treatment at 37°C ● BLM only; ▲ BLM with glycerol (**B**) Dependence of Cytotoxicity Enhancement on Glycerol Concentration in Co-treatment with 2 μg/ml BLM at 37°C for 1 h

Synergistic Cytotoxicity of BLM and Glycerol in Simultanous Treatment at 43°C

Effect of glycerol on the synergistic cytotoxicity of BLM and hyperthermia (43°C, 1 h) was examined. The survival fractions at 43°C plotted in Fig. 2 are corrected for toxicity of hyperthermia (43°C) alone. Glycerol enhanced the cytotoxicity of BLM at 43°C but it was less effective than at 37°C.

Synergistic Cytotoxicity of BLM and Glycerol in Sequential Treatments

The temporal effect on the synergistic interaction of BLM and glycerol was examined by sequential treatment of cells with BLM and glycerol for 1 h each at 37°C. The sequential treatment, regardless of the order of sequencing, produced synergistic cytotoxicities to some extent, as shown in Fig. 3. As for the dependence on the sequencing, the BLM-treatment preceding the glycerol-treatment (BLM ⟶ Gly) was more effective than the treatment in the reverse order of sequencing (Fig. 3). The treatment with BLM at 37°C was then carried out sequentially in combination with glycerol at 43°C. Similar enhancement effects were observed and the BLM-treatment preceding the glycerol-treatment was again more effective than in the reverse order of sequencing (data not shown).

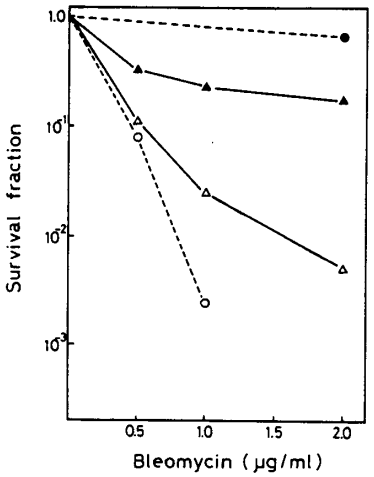

Fig. 2. Enhancement Effect of Glycerol (10%) on BLM Cytotoxicity at 43°C

Survival fractions were corrected for the toxicity of 43°C hyperthermia alone. ● BLM at 37°C; ○ BLM with glycerol at 37°C; ▲ BLM at 43°C; △ BLM with glycerol at 43°C

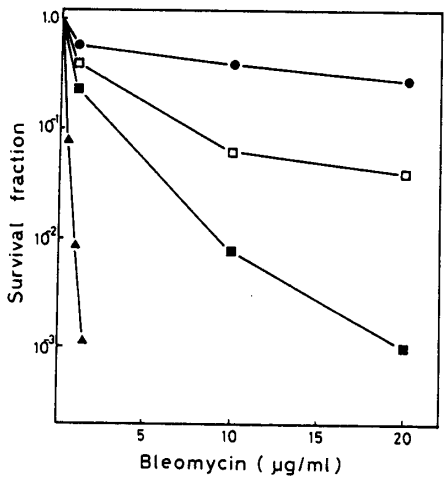

Fig. 3. Synergism in Sequential Treatments of BLM and Glycerol (10%) at 37°C for 1 h Each

● (37°C) ⟶ BLM; □ glycerol ⟶ BLM; ■ BLM ⟶ glycerol; ▲ BLM & glycerol

Cytotoxicities in Combinations of Glycerol with Adriamycin, Paraquat, Hydrogen Peroxide, or N-Methyl-N-nitrosourea

With regard to adriamycin, paraquat, and N-methyl-N-nitrosourea, there was no appreciable difference from the treatment with each chemical alone in combination with glycerol at 37°C (Fig. 4). In combination with H_2O_2, the presence of glycerol appeared to protect cells against H_2O_2-toxicity to an appreciable extent.

DISCUSSION

We have shown that BLM-toxicity was greatly enhanced by the presence of glycerol (10%) at 37°C. BLM-toxicity was enhanced by hyperthermia as previously reported, but in the presence of glycerol it was slightly reduced at 43°C. These

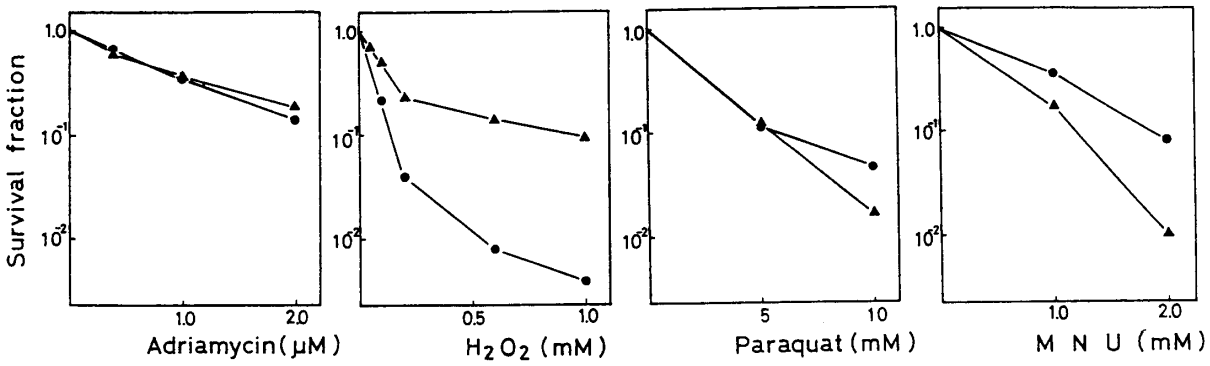

Fig. 4. Effect of Glycerol (10%) on Cytotoxicity of Adriamycin, Paraquat, Hydrogen Peroxide, and MNU
● without glycerol; ▲ with glycerol

findings indicate that glycerol protects cells from thermal injury which interacts with BLM-toxicity and that the synergism between BLM and glycerol may involve a different mechanism from that involved in the synergism between BLM and hyperthermia. In the sequential treatment, the BLM treatment preceding the glycerol treatment was more effective than in the reverse order of sequencing. This suggests that mechanisms other than the effect on the permeability of BLM may operate. There was no synergism of glycerol with any other chemicals tested in this study. Among these, glycerol remarkably reduced the H_2O_2 toxicity. This is consistent with the fact that glycerol acts as a radical scavenger. Synergism between BLM and glycerol may still be open to further investigation.

Implication for a Clinical Application of the Synergistic Combination of BLM and Glycerol

The combination of BLM and hyperthermia has been applied clinically in patients with bladder cancer by irrigating the bladder cavity with a hot BLM solution. The preirrigation of the bladder cavity with a hot aqueous ethanol solution preceding the BLM irrigation was suggested to be effective even with moderate hyperthermic temperatures.[8] The present study may provide an alternative and promising method to treat bladder cancer by irrigating the bladder cavity with a BLM solution containing 10% glycerol without hyperthermia.

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