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TEMPERATURE-DEPENDENCE OF CYTOTOXICITY OF SEVERAL GENOTOXICANTS IN CHINESE HAMSTER V79 CELLS: BLEOMYCIN, PARAQUAT, AND SOME N-ALKYL-N-NITROSOUREAS

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SUMMARY Chinese hamster V79 cultured cells were treated with bleomycin (BLM), paraquat (PQ), N-methyl-N-nitrosourea (MNU), and ACNU (an anti-cancer agent) at 30-43°C. The survival fractions normalized by those released from heat-death were plotted vs. the integrated doses of the chemical. Arrhenius analysis of the cell inactivation by the chemical was made. The results revealed that BLM and PQ exerted synergistic cytotoxicity with hyperthermia, whereas MNU and ACNU exerted temperature-dependent cytotoxicities obeying the Arrhenius law.

Since tumor cells have been recognized to be more heat-sensitive than normal cells, a large number of papers have been published on the cytocidal effect of hyperthermia alone (1) and the synergism between hyperthermia and drugs in cultured tumor cells and tumor tissues in animals (2). In principle, the cytocidal effect of chemicals such as genotoxic electrophiles and oxidants depends upon the temperature of exposure since the cytotoxicity is attributed to the chemical modifications of the cell constituents. It is, therefore, expected that the temperature dependence of cell killing may be numerically

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Abbreviations: **BLM**, bleomycin; **PQ**, paraquat; **MNU**, N-methyl-N-nitrosourea; **ACNU**, N-(2-chloroethyl)-N'-(4-amino-2-methyl-pyrimidin-5-yl)methyl-N-nitrosourea.

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described by an Arrhenius equation. In the present report, Arrhenius analysis of the cytotoxicity of 4 genotoxic chemicals is described and the synergism between hyperthermia and cytotoxicity of chemicals is briefly discussed. Only a few papers have so far dealt with Arrhenius analysis of the cytotoxicity of chemicals (3,4).

In the present study, V79 cells were treated with chemicals for 1 h at several temperatures between 37°C and 43°C. The surviving fraction of the chemical-treated cells was normalized by that of the untreated cells at the respective temperatures. This normalized fraction is regarded as the survival fraction released from cytotoxic effect of the chemical. This procedure is rationalized on the assumption that the initial cell population is homogeneous with respect to the lethal effects of both chemical and hyperthermia, i.e., the both cell populations killed by heat and survived from heat-death have the same sensitivity toward the cytotoxicity of the chemical. These surviving fractions were then plotted against the concentration-time integrated dose of chemical. From these data, an Arrhenius analysis of the cell killing was carried out.

EXPERIMENTAL

BLM (bleomycin A_2) was purchased from Nippon Kayaku Co., Ltd. (Tokyo), PQ was obtained from Sigma Chemical Co. (St. Louis, MO), ACNU was purchased from Sankyo Co., Ltd. (Tokyo), and MNU was synthesized in our laboratory.

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. After 2 days' culture, 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 5,000,000 cells/ml. This suspension (0.2 ml) was added to 0.8 ml of Dulbecco's PBS (pH 7.4) containing a chemical to be tested and incubated at a specified temperature for 1 h with gentle shaking. The cells were then collected by centrifugation and washed with the PBS. The cells were resuspended in the culture medium and diluted with PBS to appropriate volumes so that the surviving cell density became 100-200 cells/plate. The cell suspension (0.1 ml) was then seeded in 4 ml of culture medium in a 60mm culture dish.

After 7-10 days' incubation, the colonies formed were fixed, stained, and counted.

Kinetics of Cell Killing

The reliable cell survival data are obtained from the colony-forming ability of the treated cells. The dose-response curve of cell killing is characterized in many cases by a linear exponential portion (3-5) following, in some cases, a non-linear region. The kinetics of cell killing is often described in terms of the mean lethal dose, D_o, which is defined as the dose required for 37% reduction in colony formation on the linear exponential portion. On the basis of single-event Poisson statistics, D_o may be given a physical meaning representing the dose producing one lethal hit (damage) on the average in the lethal target(s) (5). The relative rate of the production of lethal hit leading to cell killing may be expressed by $1/D_o$ (1b). Since the chemicals are generally considered to disappear via first order reaction kinetics with respect to the concentration of chemical, D_o should be termed, especially for kinetical analysis of cell killing, as the concentration-time integrated dose which is formulated in terms of the disapperance rate of the chemical in question and the exposure time, as follows:

$$D_o = C_o(1-e^{-kt})/k$$

where k is the rate constant of the disappearance, C_o is the initial concentration of the chemical, and t is the duration of exposure.

Temperature dependence of chemical events is, in general, well described by Arrhenius equations. For the relation between cell killing and exposure temperature, the logarithmic $1/D_o$ is plotted as a function of the inverse of the absolute temperature.

$$\ln(1/D_o) = \ln A - Ea/RT$$

where A is a constant, Ea is the apparent activation energy for cell killing, R is the gas constant, and T is the absolute temperature for exposure. The dimension of $1/D_o$ is the same as that of the second order rate constant, 1/M (time). Ea can be calculated from the slope of the Arrhenius plot.

RESULTS AND DISCUSSION

The chemicals investigated in this study are bleomycin (BLM) and paraquat (PQ) which are known to generate active oxygen species (6,7), N-methyl-N-nitrosourea (MNU) which is a simple genotoxic alkylating agent, and N-(2-chloroethyl)-N'-(4-amino-2-methylpyrimidin-5-yl)methyl-N-nitrosourea (ACNU) which is an anti-cancer agent. These agents do not require any specific enzymic activations for their cytotoxicity. For BLM and PQ, no appreciable decomposition was observed during 1 h's incubation in the exposure medium, whereas MNU and ACNU disappeared by spontaneous hydrolysis at the following rates.

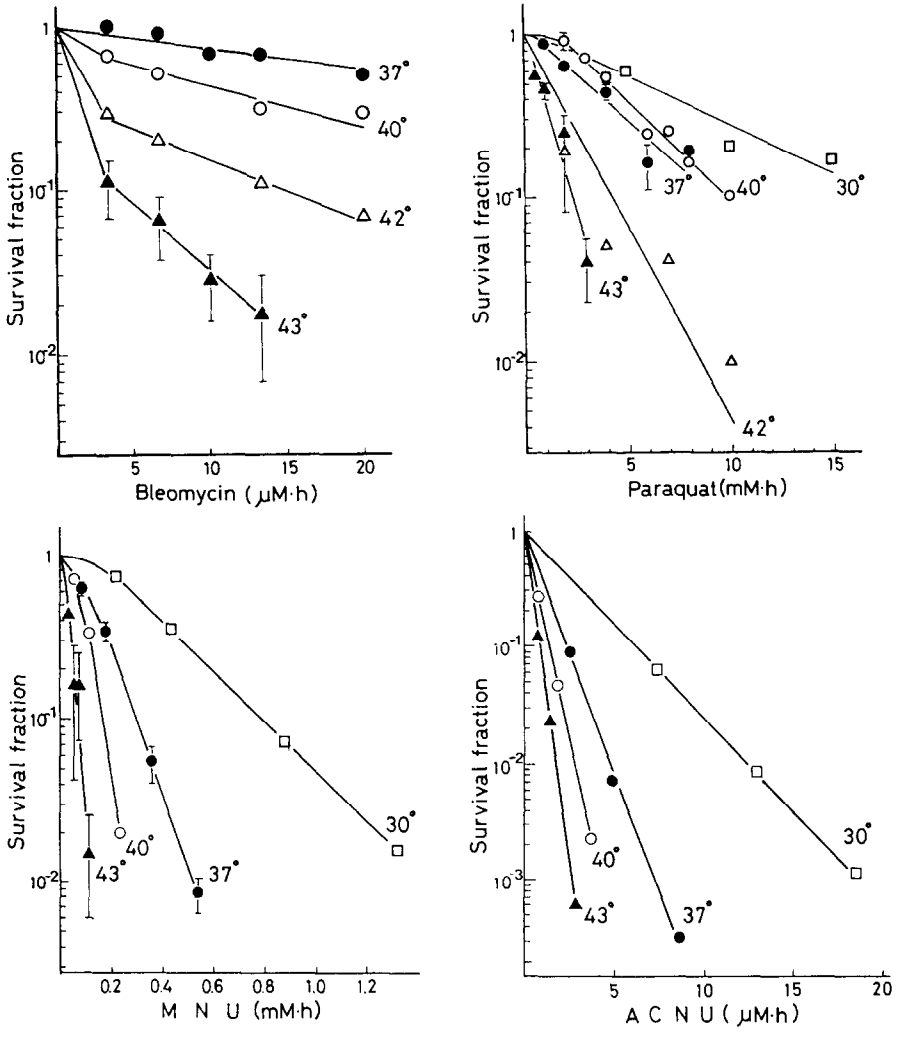

Figure 1. Plots of survival fractions vs. integrated doses of BLM, PQ, MNU, and ACNU

The rates were determined by UV spectroscopy (8). Pseudo-first order rate constants at pH 7.4 for MNU: 1.95/h at 30°C, 5.53/h at 37°C, 8.52/h at 40°C, and 13.01/h at 43°C; those for ACNU: 0.668/h at 30°C, 1.65/h at 37°C, 2.40/h at 40°C, and 3.47/h at 43°C.

The plots of survival fractions vs. integrated dose of chemicals are shown in Fig. 1. Each data point is the average from more than three independent experiments. The D_o value was read from the slope of the linear portion of each dose-response

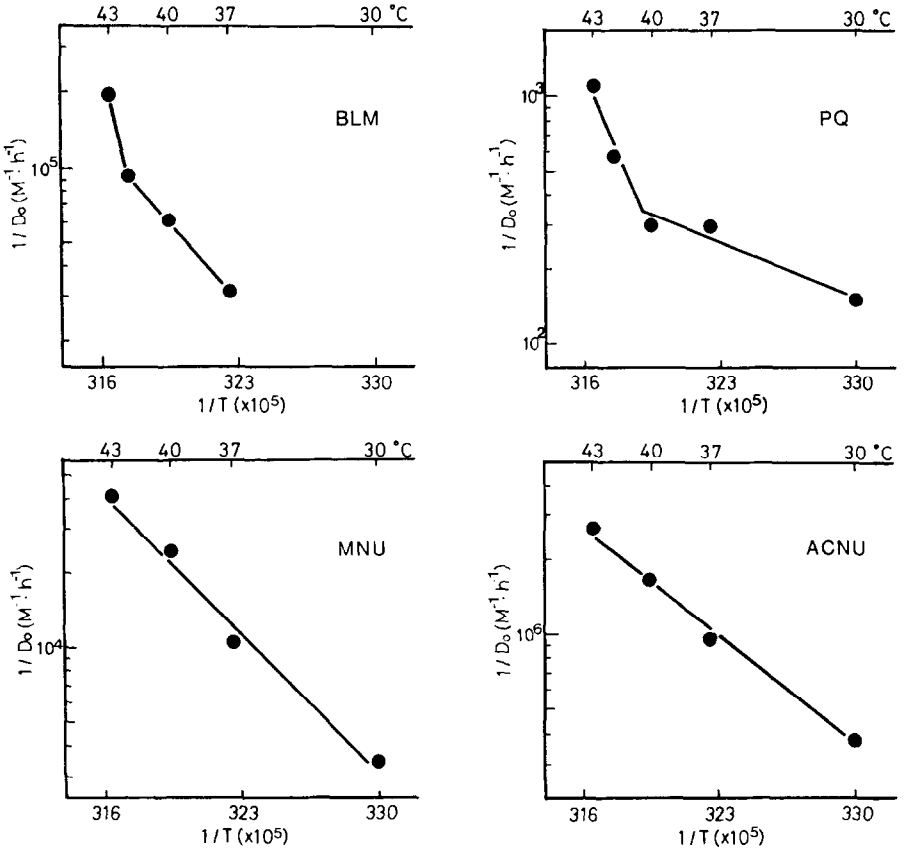

Figure 2. Arrhenius plots for cell inactivation by BLM, PQ, MNU, and ACNU

curve. With regard to BLM, an upward-concave region is seen in the dose-response curve (9). Aside from the origin of this concave region, the linear portion was tentatively taken into consideration in this study.

Fig. 2 shows the Arrhenius plots of $\log(1/D_o)$ versus $1/T$. A break point was clearly found on Arrhenius plots of BLM and PQ between 30 and 43°C, whereas MNU and ACNU gave almost linear plots. The apparent activation energies for cell inactivation were calculated from the slopes of the plots: 44 and 167 Kcal/mol for BLM, 16 and 82 Kcal/mol for PQ, and 40 Kcal/mol for MNU, and 29 Kcal/mol for ACNU. The values are regarded as the apparent activation energies for the cell inactivation induced by the toxicity of chemicals alone or with hyperthermic synergism.

As shown in Fig. 2, cytotoxic synergism between hyperthermia and BLM or PQ was clearly indicated. BLM is known to show a synergistic cytotoxicity under hyperthermic conditions (10). A synergistic effect of heat on the chemical toxicity or alternatively, an effect of the chemical on the hyperthermic toxicity might be realized. On the other hand, both alkylating agents, MNU and ACNU, gave normal linear Arrhenius plots. These results suggest that the origin of synergism might possibly be related to oxygen toxicity.

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