THERMAL ENHANCEMENT OF DNA DAMAGE BY AN ALKYLATING AGENT IN HUMAN CELLS

Burt V. Bronk[1], R. J. Wilkins, and James D. Regan

Biology Division, Oak Ridge National Laboratory[2]
Oak Ridge, Tennessee 37830

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SUMMARY. Human skin cells were incubated at various temperatures during and after treatment with methyl methanesulfonate and the number of single-strand breaks introduced into the cellular DNA then estimated by alkaline sucrose sedimentation. Elevation of temperature above 37° greatly enhanced damage to the DNA caused by methyl methanesulfonate. Inactivation of an essential step in the repair of DNA was indicated by the observation that rejoining of breaks in the DNA was halted above a critical temperature (about 41.5°). Enhancement of damage to DNA increased with temperature, especially above 42°. Similar results were obtained for Chinese hamster cells. A correlation of these results with cell viability is discussed.

Mammals normally regulate their body temperature to within a narrow interval around 37° and have therefore been able to evolve by selecting for mutations which improve body function at the expense of wide thermal tolerance. Thus changes in body temperature of more than a few degrees cause a serious deterioration in function. On the cellular level, incubation at temperatures away from the optimal 37° is known to greatly slow the cell cycle (1) and, although most cells will survive at 42° for several hours, prolonged incubation above this temperature results in cell death (2,3). In this respect mammalian cells resemble temperature-sensitive bacterial mutants which cannot grow at high temperature (usually about 42° or above) because an essential protein has been rendered thermally unstable (4).

One may ask whether thermal inactivation of an essential recovery mechanism might be found to enhance nonthermal damage to the cell at elevated temperatures. Such a synergistic effect does indeed occur when Chinese hamster cells are exposed to x-rays at elevated

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[1] Present address: Physics Department, Clemson University, Clemson, South Carolina 39631.

[2] Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

temperature (5, 6). Radiosensitivity, as measured by loss of colony-forming cells, is enormously increased and split-dose experiments indicated that inhibition of repair of sublethal damage occurs at elevated temperature. In this paper we present similar synergistic effects in human cells treated with methyl methanesulfonate (MMS), an alkylating agent which is believed to kill cells primarily through damage to cellular DNA (7).

MATERIALS AND METHODS

Human diploid fibroblasts derived from normal foreskin (HSWP cells, doubling time about 26 hours) were employed in these experiments. Experiments were performed on cultures with less than ten passages from the original culture. A Chinese hamster fibroblast line (8) (CH cells, doubling time about 12 hours) derived from CHEF 125 was used for comparison and gave similar results to those for the human cells. The cells were grown in monolayers in 25 cm^2 plastic (T-30) flasks (Falcon) in Eagle's minimal medium with 15% fetal calf serum plus the nonessential amino acids (9).

Cellular DNA was labeled by overnight incubation (about 20 hours) in [^{3}H]dThd (2 μCi/ml) or $^{32}PO_4$ (10 μCi/ml). Labeling medium was removed and replaced with 5 ml regular growth medium for about 4 hours prior to the experiments. During thermal treatment, flasks were sealed and immersed in water after the medium was first brought to equilibrium with an atmosphere containing 2% CO_2 at 37°. Temperatures were held to within ± .15° of the nominal value. Cells were equilibrated at the treatment temperatures for about one-half hour before addition of MMS (freshly diluted in distilled water to concentrations such that 0.05 to 0.1 ml added to 5 ml of medium gave the desired concentration). Cells were treated at concentrations of from 5 X 10^4 to 2 X 10^5 cells per flask, and at the end of treatment the flasks were either chilled in ice for posttreatment studies or washed and then incubated in fresh medium. The cells were collected after decanting the medium by breaking the flat top face of the flask and scraping with a rubber policeman into 2 ml of an EDTA solution (10). The cells were then concentrated by centrifugation and resuspended in the EDTA solution at a concentration of 2 X 10^5/ml for layering on gradients. The number of single-strand breaks was estimated by sedimentation in 5 to 20% alkaline–sucrose gradients as described previously (10, 11) (see Fig. 1). Weight average molecular weights were computed after omitting the bottom fraction and the top five fractions from the gradient. Controls were run through a thermal schedule appropriate to each experiment to obtain control sedimentation profiles (see Fig. 2a) and untreated weight average molecular weight, M_o, for comparison with treated molecular weight, M_t, for the given experiment. When convenient, treated and control DNA were run on the same gradient by using two

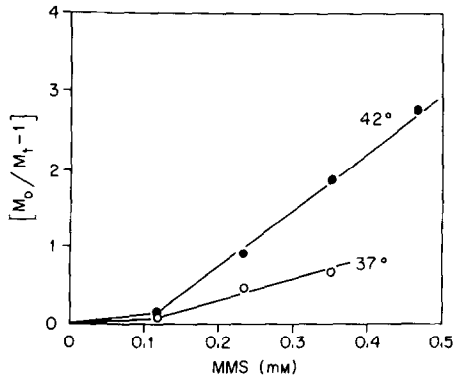

Fig. 1. HSWP cells were treated with MMS for 6 hours. Weight average molecular weights of single-stranded DNA were determined for treated (M_t) and untreated (M_o) cells at 37° or 42° and are used to determine relative number of strand breaks. All sedimentations were done as follows. About 10,000 cells were gently mixed into a 0.2-ml overlay of 1.0 M NaOH on top of a 3.6-ml alkaline 5–20% sucrose gradient in the polyallomer tubes of a Beckman SW-56 rotor. After 1 hour, the samples were spun at 30,000 rpm for 180 min at 20°. A hole was punched in the bottom of each tube and approximately 28 fractions were collected on Whatman No. 17 paper strips (12) for determining radioactivity distribution by counting (radioactivity was from 0.5 to 2 cpm of TCA insoluble material per cell) in a toluene based scintillator, with molecular weights calculated (13) as proportional to $(S^{o}_{20,w})^{2.62}$ using T4 phage DNA as a standard (11, 14).

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labels (^{3}H and ^{32}P). Possible artifacts due to use of a given label were eliminated by repeating experiments after interchanging nuclides in treated and untreated cells. Experiments reported were limited to those for which thermal treatment alone* gave a normal looking sedimentation profile and an M_o which was usually in the range $1.2 \times 10^8 - 1.4 \times 10^8$ daltons for the conditions of the experiment.†

Cell viability‡ for HSWP cells was determined after MMS treatment followed by a wash and incubation in fresh medium for 24 hours. Attached cells were then removed with trypsin. Viable cells (as determined by dye exclusion of 0.2% erythrocin B) were then counted in a haemocytometer.

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*A synergistic effect was observed in CH cells for radioactive (particularly ^{32}P) disintegrations combined with temperatures over 41° which led to degradation of DNA. Such effects were eliminated for these cells by keeping duration of exposure of temperatures above 37° to about 2 hours or less.

†During the initial exposure to high temperatures, M_o increased to a maximum of about 1.6×10^8 daltons. At 46°, for example, this value was reached after 30 min incubation for HSWP cells.

‡The more desirable method of counting colony-forming units was not feasible because of the low plating efficiency of the human diploid fibroblasts.

RESULTS AND DISCUSSION

Damage to the DNA is indicated by an increased value of $[(M_o/M_t) - 1]$ which gives a measure of the average number of single-strand breaks per unit strand length induced by MMS treatment. In Fig. 1 we show the results of a 6-hour treatment of HSWP cells with various concentrations of MMS at 37° and 42°. A rapid increase in DNA breakage, after some critical level of damage, was typically observed in our MMS experiments. While the damage is not measurably different at low concentrations, the number of breaks induced at the high temperature increases much more rapidly at 42° than at 37° as the initial concentration of MMS is increased.[§]

In Fig. 2 we see a sequence of sedimentation profiles. These suggest that the great increase of the number of single-strand breaks for temperatures above 42° may be due to a loss of repair function. In Fig. 2a a typical control profile[‖] is shown, as well as the profile for treatment with 0.93 mM concentration of MMS for 1 hour at 37° ($M_t \approx 0.6 \times 10^8$ daltons) and the much lower molecular weight ($M_t \approx 0.3 \times 10^8$ daltons) for the treatment which is identical but at 42°. In Fig. 2b we see that when the 37° treatment is followed by posttreatment incubation at 37° for 1.5 hours, over 2/3 of the strand breaks rejoin ($M_t \approx 0.95 \times 10^8$ daltons). When the same treatment is followed by incubation at 42° for 1.5 hours, the profile moves to the right ($M_t \approx 0.4 \times 10^8$ daltons) and is similar to that after the 1 hour treatment at 42°. This indicates that strand rejoining is inhibited at 42° and that some further breakage has occurred. Finally, at 3 hours postincubation following the 1-hour 37° treatment (Fig. 2c), we see that the cells postincubated at 37° have a sedimentation profile undistinguishable from a control profile as shown in Fig. 2a and that the weight averaged molecular weight has returned to a value, $M_t \approx 1.3 \times 10^8$ daltons. The cells postincubated at 42° following the same 37° treatment (Fig. 2c) have a profile at 3 hours posttreatment incubation essentially identical ($M_t \approx 0.45 \times 10^8$ daltons) to that they had at 1.5 hours posttreatment incubation (Fig. 2b). Longer posttreatment incubations at the same temperatures have no further effect on the profiles. Thus repair as measured by strand-break rejoining occurs at 37° but is inhibited at 42°. This is probably the cause of greatly enhanced damage to DNA by MMS during prolonged treatment at high temperatures.

[§]A similar result, although at somewhat higher doses of MMS, was observed when the exposure time was reduced to 130 min. In this case the number of breaks for 42° increased much more rapidly than for 37° with concentration of MMS when the concentration was greater than 0.4 mM.

[‖]The control profile obtained by following the thermal schedule for a given experiment, but without MMS, was essentially identical to that shown in Fig. 2a for all experiments reported here.

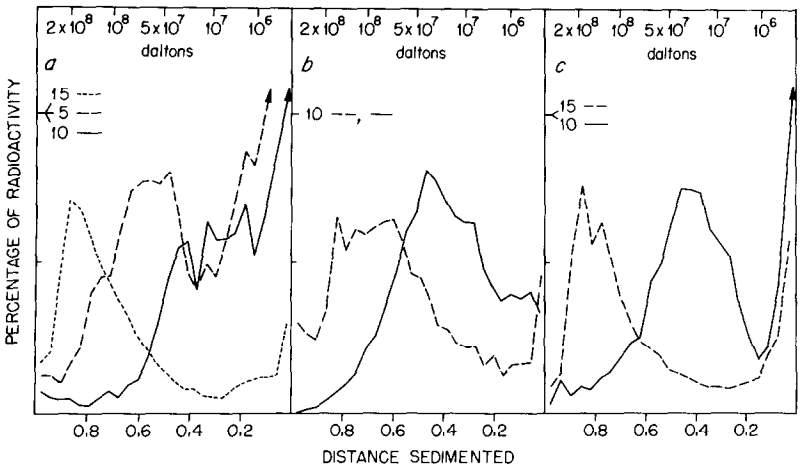
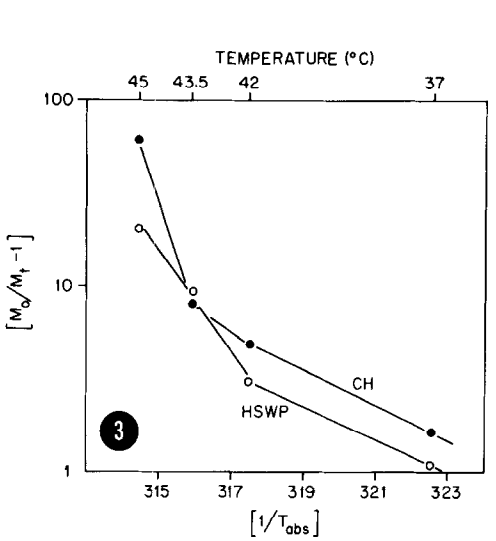


Fig. 2. Sedimentation profiles of DNA from HSWP cells treated for 1 hour with 0.93 mM concentration of MMS: (a) Untreated control, ---; treated at 37° for 1 hour, — —; treated at 42° for 1 hour, ——. (b) Treated at 37° for 1 hour, washed, then incubated, in fresh medium at 37° for 1.5 hours, — —; treated at 37° for 1 hour, washed, then incubated in fresh medium at 42° for 1.5 hours, ——. (c) Treated at 37° for 1 hour, washed, then incubated in fresh medium at 37° for 3 hours, — —; treated at 37° for 1 hour, washed, then incubated at 42° for 3 hours, ——. Posttreatment washings were with Hanks' buffered salt solution at about 37°. The untreated control shown in (a) was typical for exposures of HSWP to 42° for up to 6 hours or 46.4° for up to 195 min.

Other experiments indicate that if the damage, as indicated by single-strand breaks is light, some rejoining may take place at 42°, but this is always less than the amount that would take place with posttreatment incubation at 37°.

The relative number of single-strand breaks is plotted against the inverse of the absolute temperature in Fig. 3. The logarithmic scale of the number of strand breaks makes this an "Arrhenius type" plot, but the increase of the negative slope with decreasing inverse temperature indicates that the increasing rate of a single chemical reaction is not the cause of enhancement of DNA damage with temperature. More likely is potentiation of damage due to loss of an essential repair function which is present at 37°, combined with an increase in degradation due to the normal Arrhenius effect on nuclease activity at increased temperature. Possibly a repair enzyme is inactivated by a change in conformation of the protein mediated by the input of thermal energy. That such inactivating conformation changes may be common is suggested by the ubiquity of temperature-sensitive mutations in bacteria which cause loss of an enzyme's activity only above a critical temperature for which the activity is still normal in the wild type (4). It is important to note that CH and HSWP cells show similar plots in Fig. 3, indicating that the phenomenon is probably universal for mammalian cells.

The above described results are even more interesting because elevation of temperature



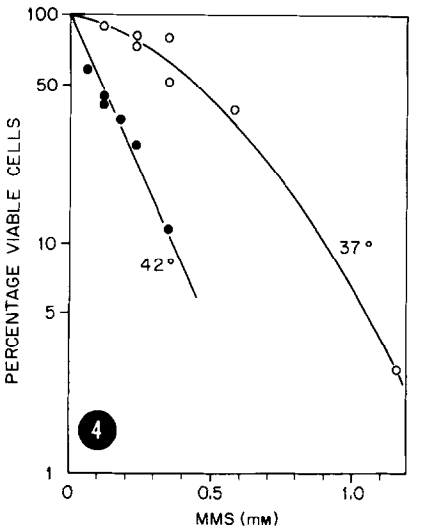


Fig. 3. HSWP and CH cells were treated with a 0.23 mM concentration of MMS for 2 hours at the indicated temperatures.

Fig. 4. HSWP cells were treated at 37° and 42° for 6 hours with various concentrations of MMS, washed, then incubated in fresh medium for 24 hours at which time viability was obtained.

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above 37° for mammalian cells enhances lethality of MMS treatment as determined by survival of colony-forming units for Chinese hamster cells (15). We demonstrated thermal enhancement of lethality for human cells with viability studies. HSWP cells were treated with various concentrations of MMS (up to 0.33 mM) for 6 hours during incubation at 37° or 42°. Typical viability curves are shown in Fig. 4. The response to MMS is roughly logarithmic, with a shoulder apparent for treatment at 37° but not at 42°. A typical experiment gave a D_{37} of 0.15 mM MMS at 42°, while at 37° the D_{37} is somewhat greater than 0.5 mM. Hence it takes a much greater dose of MMS to kill the cells at 37° than at 42°. We find therefore that thermal enhancement of lethality is extended to human cells. In the present case of treatment of human cells with the alkylating agent, MMS, the enhanced lethality at elevated temperatures is correlated with a greater number of DNA strand breaks.[#]

The present experiments suggest that chemotherapeutic treatment of tumors can be

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[#]With x-irradiation, for which increased lethality has been observed for Chinese hamster cells at elevated temperatures (5, 6), this is not the case. We ran x-ray experiments on CH cells with thermal schedules similar to those reported here for MMS experiments, but the number of strand breaks did not increase at elevated temperatures with doses up to 1400 rads at dose rates of about 3.8 rad/min.

rendered more effective by mild heating of the tumor region.[¶] This is particularly attractive since localization of lethality is difficult with chemical treatment.

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[¶]Thermal treatment as an adjuvant to other forms of therapy could lead to radical improvement of therapy if malignant cells prove to be more sensitive than normal cells. Differences in response to thermal treatment alone have been noted (see ref. 16 for further references) for tumor and normal cells.