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Enhancement of 1,3-Bis(2-chloroethyl)-1-nitrosourea Resistance by γ-Irradiation or Drug Pretreatment in Rat Hepatoma Cells[1]

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ABSTRACT

Treatment of rat hepatoma cells (H4 cells) with various DNA-damaging agents increases the number of O^6-methylguanine-DNA-methyltransferase (transferase) molecules per cell. Because the cellular resistance to chloroethylnitrosoureas depends on the number of transferase molecules, we studied the influence of pretreatment with γ-irradiation, *cis*-dichlorodiammineplatinum(II), or 2-methyl-9-hydroxyellipticinium on the sensitivity of H4 cells to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). The BCNU resistance depends on the γ-ray dose and increases with time after irradiation: it is maximum when the drug is added 48 h after irradiation, which corresponds to the maximum enhancement of the transferase activity in the cells. Pretreatment with a single dose of *cis*-dichlorodiammineplatinum(II) or 2-methyl-9-hydroxyellipticinium also increases the cellular resistance to BCNU. This resistance is not due to a modification of the alkylation of the cellular DNA in the pretreated cells but is related to the increased transferase activity, as it is no longer observed when this activity is depleted by incubating the pretreated cells with the free base O^6-methylguanine before BCNU treatment. These results suggest that tumor cells surviving after γ-irradiation or drug treatment may become resistant to chemotherapy with chloroethylnitrosoureas.

INTRODUCTION

BCNU[3] is a haloethylnitrosourea widely used in cancer therapy, especially in the treatment of lymphomas and brain tumors. Several reports showed that the biological activity of this drug is due to the alkylation of the O^6-position of guanine in the cellular DNA, resulting in the formation of DNA cross-links (1), which are probably the lethal lesions formed by the haloethylnitrosoureas (2–4). DNA repair mechanisms play an important role in BCNU-induced cytotoxicity. Resistance to BCNU is correlated with transferase activity (5), and inactivation of this protein by either MNU (6) or O^6-alkylguanine pretreatment (7–11) increases cell sensitivity to nitrosoureas.

We previously showed that the pretreatment of H4 cells (rat hepatoma cells) with various chemical or physical agents that damage the cellular DNA increased the number of transferase molecules per cell (12, 13). Because the problem of tumor resistance to chemotherapeutic agents is important in cancer therapy (14), we investigated whether this enhanced repair activity had biological consequences and might result in the appearance of resistant cells. In this report, we show that the BCNU resistance of H4 cells is increased when the cells are pretreated with either ionizing radiation or chemical compounds (*cis*-DDP or NMHE) under conditions that increase transferase activity.

MATERIALS AND METHODS

Cell Culture. H4 cells, epithelial cells derived from a rat hepatoma (15), were grown in Dulbecco's medium supplemented with 5% fetal calf serum, 5% horse serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37°C in a CO_2-humidified atmosphere. All media were obtained from GIBCO. The doubling time for H4 cells was 14 h.

Irradiation and Drug Treatments. Exponentially growing cells were irradiated with a ^{60}Co γ-ray source, yielding a dose rate of 1.5 Gy/min. *Cis*-DDP (Roger Bellon, Paris, France) and 2-methyl-9-hydroxyellipticinium (Institut Pasteur, Paris, France) were dissolved in water. Incubations with these compounds were run for 1 h in complete medium at 37°C, 24 h after seeding 3.5×10^6 cells in 75-cm^2 flasks.

Depending on the experimental protocol, BCNU was added either immediately or at different times after irradiation or pretreatment with either *cis*-DDP or NMHE. BCNU was dissolved at 10 mg/ml in ethanol and then mixed to the appropriate concentration in culture medium. Cells were incubated for 1 h with BCNU and then washed with Earle's balanced salt solution and trypsinized. Aliquots of cell suspension were plated for survival and grown until the appearance of clones (15). The plating efficiency of control H4 cells was 75 to 85%.

Determination of O^6-Methylguanine-DNA-methyltransferase Activity. This activity was determined by incubating cell extracts with [^{3}H]MNU-treated DNA and measuring the remaining amount of O^6-methylguanine residues in the substrate, as already described (16).

Analysis of DNA Alkylation Products. Cells were irradiated (3 Gy) or incubated for 1 h with *cis*-DDP (5 μM), grown for 48 h in normal medium, and then incubated for 30 min with [^{3}H]MNU (Amersham; 0.5 mCi/ml, 1.5 Ci/mmol). The cells were then washed, trypsinized, and lysed by the addition of sodium dodecyl sulfate, and the cellular DNA was separated by centrifugation in CsCl gradients, as already described (17). The DNA was dialyzed against potassium phosphate (1 mM, pH 7.6) and hydrolyzed as described by Frei *et al.* (18). The alkylated bases were separated by high pressure liquid chromatography (18) using a C_{18}-μBondapak column (Waters). The amount of DNA-P was calculated as described by Medcalf and Lawley (19), and the amount of alkylated bases was determined by scintillation counting.

RESULTS

Transferase Activity in γ-irradiated or Drug-treated Cells. As we have already shown (12), when H4 cells are γ-irradiated (3 Gy) or incubated for 1 h with *cis*-DDP (5 μM) or NMHE (2.5 μg/ml), the transferase activity increases. The number of transferase molecules begins to increase 7 h after the treatment, reaches a maximum value between 48 and 72 h, and then decreases to the control value after 120 h (12). However, when the cells are treated with repeated doses (*e.g.*, when they receive a γ-ray dose every 48 h), the number of transferase molecules is enhanced for the duration of the treatment (data not shown). Furthermore, as shown in Fig. 1, the transferase activity can be depleted either in control or in treated cells by adding the free base O^6-methylguanine (1 mM) to the culture medium for 3 h.

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[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MNU, *N*-methyl-*N'*-nitrosourea; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); NMHE, 2-methyl-9-hydroxyellipticinium; transferase, O^6-methylguanine-DNA-methyltransferase; D_e, dose required to reduce survival by the factor 1/e, calculated from the linear part of the survival curves and corresponding to a 37% survival.

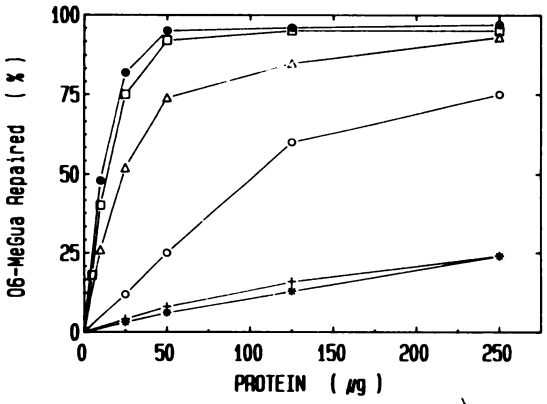

Fig. 1. Removal of O^6-methylguanine (*O6-MeGua*) from alkylated DNA by H4 cell extracts. [^{3}H]MNU-treated DNA was incubated with extracts from control (○), γ-irradiated (3 Gy) (●), *cis*-DDP-treated (5 μM, 1 h) (△), or 2-methyl-9-hydroxyellipticinium-treated (2.5 μM, 1 h) (□) cells. The transferase activity was measured in cells grown for 48 h in normal medium after these treatments. Additionally, control (+) or preirradiated cells (3 Gy) (*) were incubated for 3 h with O^6-methylguanine (1 mM) before transferase activity determination.

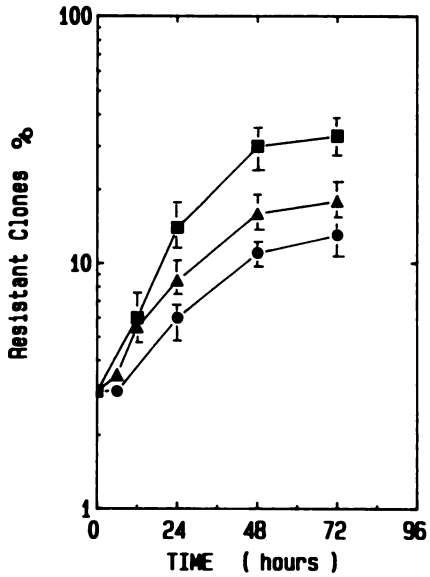

Fig. 2. Effect of postirradiation time on the BCNU resistance of H4 cells. The cells were γ-irradiated and grown for 0, 6, 12, 24, 48, or 72 h. They were then trypsinized, and aliquots were seeded in BCNU-containing medium (20 μg/ml). The cultures were incubated at 37°C until the appearance of clones. Results are expressed as the percentage of surviving clones and are corrected for γ-ray survival. Cells were irradiated with a dose of 3 Gy (●), 5 Gy (▲), or 7.5 Gy (■).

Cell Sensitivity to BCNU. To determine if ionizing radiation enhances the BCNU resistance of H4 cells, the cells were irradiated with 3, 5, or 7.5 Gy, grown for different periods, and then trypsinized. Aliquots of cell suspension (10^3 to 10^4 cells) were seeded in BCNU-containing medium (20 μg/ml), and the cells were grown in the presence of this drug until the appearance of clones. The number of resistant colonies increased with the time separating the irradiation and the drug treatment and with the γ-ray dose (Fig. 2). The maximum increase was observed when the drug was added 48 h after the irradiation, which corresponds to the maximum enhancement of transferase activity in preirradiated cells (12).

However, it was determined that these colonies were resistant to BCNU and were not the result of a selection of radioresistant cells. Therefore, BCNU-resistant clones were randomly selected and grown, and their sensitivity to γ-rays was determined. The survival curve parameters for these clones were a D_o of 1.5 to 1.55 Gy and an extrapolation number of 2.9 to 3.0, which correspond to the values already established for exponentially growing H4 cells (15).

To determine the resistance of irradiated cells to BCNU, the cells were grown for 48 h in normal medium after γ-irradiation (3 or 5 Gy) and then were incubated for 1 h with increasing BCNU concentrations. Fig. 3 shows the survival curves of cells that were or were not irradiated before BCNU treatment; the irradiated cells were more resistant to the drug than were control cultures. There was similar BCNU resistance in cells treated for 1 h with *cis*-DDP or NMHE and incubated 48 h later with BCNU (Fig. 4).

Results summarized in Table 1 show the number of transferase molecules per cell measured 48 h after the various pretreatments and the D_o for BCNU-treated cells. The BCNU resistance increases with the transferase activity in the pretreated cells.

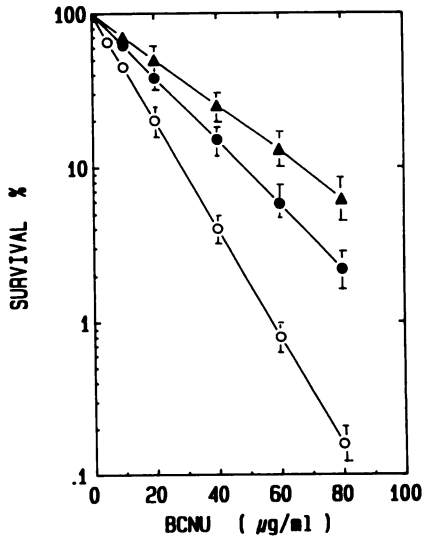

Fig. 3. Survival curves for H4 cells that were or were not γ-irradiated before BCNU treatment. Exponentially growing cells were γ-irradiated, grown for 48 h, and then incubated for 1 h with BCNU. The cells were rinsed and trypsinized, and aliquots were plated for survival determination. Data are corrected for γ-ray-induced cell killing: (○) unirradiated cells; cells irradiated with 3 Gy (●) or 5 Gy (▲). Results are the mean values of 3 separate experiments.

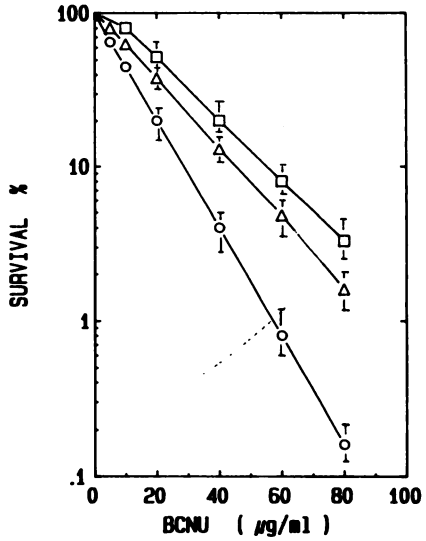

Fig. 4. Effect of *cis*-DDP or 2-methyl-9-hydroxyellipticinium pretreatment on cell resistance to BCNU. Exponentially growing H4 cells were incubated for 1 h with either *cis*-DDP (5 μM) or NMHE (2.5 μg/ml), rinsed, grown for 48 h in normal medium, and then incubated for 1 h with increasing amounts of BCNU. Results are corrected for the pretreatment-induced cell killing. These are the mean values of two separate experiments: survival of control cells (○) and of cells pretreated with *cis*-DDP (5 μM) (△) or NMHE (2.5 μg/ml) (□).

Table 1 *Influence of various pretreatments on the number of transferase molecules per H4 cell and on the D_o for BCNU-treated cells*

The number of transferase molecules and the resistance to BCNU were measured 48 h after γ-irradiation or after incubation (1 h) with *cis*-DDP or NMHE.

Cell pretreatment	Survival of pretreated cells (%)	No. of transferase molecules/cell[a]	D_o for BCNU-treated cells[b] (μg/ml)
None	100	54,000 ± 6,600	12.1 ± 1.2
γ-rays			
3 Gy	35	285,000 ± 27,600	22.3 ± 2.5
5 Gy	12	312,000 ± 25,000	28.8 ± 2.4
cis-DDP (5 μM)	55	207,000 ± 12,200	18.0 ± 1.7
NMHE (2.5 μg/ml)	30	252,000 ± 6,000	21.8 ± 2.2

[a] Mean ± SD of 3 separate experiments (from Ref. 12). The values were calculated from the linear part of the curves obtained by incubating increasing amounts of cell extracts with [^{3}H]MNU-treated DNA, as described in Fig. 1.
[b] Calculated from the survival curves.

Table 2 *Amount of methylated bases in [^{3}H]MNU-treated H4 cells pretreated with γ-rays or cis-DDP or without pretreatment*

H4 cells were irradiated (3 Gy) or incubated for 1 h with *cis*-DDP (5 μM) and were grown for 48 h in normal medium. They were then incubated with [^{3}H]MNU (0.5 mCi/ml) for 30 min. For details, see "Materials and Methods."

Cell pretreatment	μmol of methylated bases/mol DNA-P		
	N^3-methyladenine	N^7-methylguanine	O^6-methylguanine
None	0.75	7.85	0.98
γ-rays (3 Gy)	0.74	7.58	0.96
cis-DDP (5 μM)	0.75	7.91	0.97

The cell resistance to BCNU could also be due to a modification of the amount of drug taken up by the cells and to a decrease in the alkylation of the cellular DNA in the pretreated cultures. Therefore, H4 cells were either irradiated (3 Gy) or treated for 1 h with *cis*-DDP (5 μM), grown for 48 h in normal medium, and then incubated with [^{3}H]MNU for 30 min. The amount of alkylated bases was determined, and the results show that the extent of methylation was similar in control and in pretreated cultures (Table 2). Although this experiment was run using [^{3}H]MNU, as the radiolabeled chloroethylnitrosourea was not available, it suggests that a modification of the alkylation rate is probably not involved in the cellular resistance.

To establish that this BCNU resistance was actually due to the increased transferase activity, the cells were or were not γ-irradiated were grown for 48 h, and then were incubated in the presence of O^6-methylguanine (1 mM) for 3 h before BCNU treatment. Incubation with this modified base was not cytotoxic under our experimental conditions but depleted the transferase activity in the cells (Fig. 1). The sensitivity to BCNU is similar whether cultures are preirradiated or not if they are incubated with the methylated base and is greater than in cells treated with the nitrosourea in the absence of O^6-methylguanine (Fig. 5). The D_o, calculated from the survival curves, is 12.1 μg/ml for control cells and 7.5 μg/ml for cells preirradiated or not and then incubated with the free base before BCNU treatment.

DISCUSSION

The present study shows that pretreatment with physical or chemical agents enhances the resistance of H4 cells to BCNU-induced cytotoxicity. This increased BCNU resistance parallels the induction of the transferase activity in the pretreated cells, and these results are consistent with previous work showing that cell sensitivity to haloethylnitrosoureas is related to repair of the O^6-chloroethylguanine residues (5). The magnitude of the effect depends on the time interval between radiation and

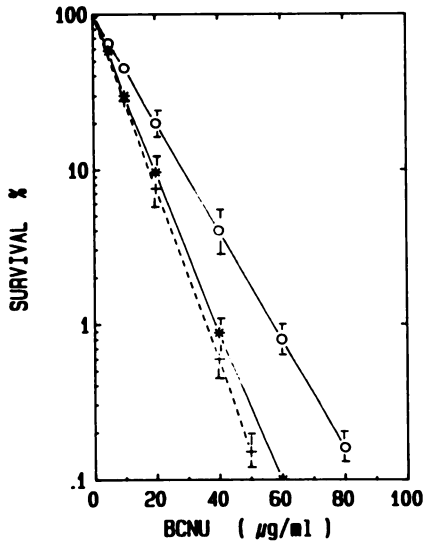

Fig. 5. Influence of O^6-methylguanine on BCNU cytotoxicity in preirradiated H4 cells: survival of H4 cells treated for 1 h with increasing BCNU concentrations in normal medium (○); survival of H4 cells preirradiated (3 Gy) (*) or not preirradiated (+), grown for 48 h, and incubated with O^6-methylguanine (1 mM) for 3 h before BCNU treatment.

BCNU treatment: the effect is maximum 48 h after pretreatment, and this length of time corresponds to the maximum enhancement of transferase activity in H4 cells treated with γ-rays or chemical compounds (12). Further evidence supporting the fact that the BCNU-enhanced resistance in the pretreated cells is actually due to the induced transferase activity is the observation that adding O^6-methylguanine to the culture medium, which depletes the amount of cellular transferase, abolishes the response.

Although different mechanisms are involved, resistance to other drugs and induction of proteins also occur in irradiated cells. γ-Irradiation induces resistance to methotrexate in Chinese hamster ovary cells and in 3T6 murine cells (20). γ-Irradiation of RF1 tumor cells increases their resistance to 5-fluorouracil, methotrexate, and adriamycin (21). Treatment of human sarcoma cells with X-rays also increases the level of tumor necrosis factor (TNF-α) mRNA and the production of TNF-α protein (22).

We previously showed that the pretreatment of H4 cells with different agents that damage the cellular DNA increased the number of transferase molecules per cell (12) and that this increase seemed related to modifications of the cellular DNA structure (23). Therefore, our results suggest that the BCNU resistance might be increased by pretreating the cells with a variety of agents other than those reported in this paper (*e.g.*, UV light or alkylating agents). Whatever the molecular mechanism of this induction, BCNU resistance might appear during cancer treatment when haloethylnitrosoureas are delivered after radiotherapy or during the course of chemotherapy or radiotherapy.

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