Eur J Cancer Clin Oncol, Vol. 24, No. 12, pp. 1861–1868, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

Differential Sensitivity of Histone Acetylation in Nitrogen-Mustard Sensitive and Resistant Cells. Relation to drug uptake, formation and repair of DNA-interstrand cross-links

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Abstract—*Cultivation of Ehrlich-ascites tumor cells in the presence of* N-*mustard leads to a selection of cells with a defective choline carrier. As* N-*mustard employs the choline carrier for transport, this results in reduced drug uptake and in a decrease in drug sensitivity which is specific for* N-*mustard. Walker carcinoma cells with a stable pleiotropic resistance to a variety of alkylating agents and adriamycin exhibit no evidence for an impaired drug transport and show the same frequency of DNA-interstrand cross-links as the sensitive parental line. Both sensitive and resistant Walker cells exhibit equal capacities for repair of* N-*mustard induced DNA-interstrand cross-links. The inhibition of histone acetylation by* N-*mustard, however, was found to be significantly lower in the resistant Walker or Ehrlich cells compared to sensitive counterparts. Although the difference between* N-*mustard concentrations leading to half maximal inhibition of histone acetylation in sensitive and resistant cells is considerably smaller than the difference between* N-*mustard doses required for half maximal inhibition of cell proliferation the data suggest that—besides DNA–DNA cross-linking—the inhibition of histone acetylation has to be considered as an important alternative mechanism responsible for the cytotoxic activity of alkylating agents. Inhibition of histone acetylation is not due an accelerated deacetylation and is predominantly expressed in chromatin fractions soluble in* 0.1 M NaCl *after digestion with micrococcal nuclease.*

INTRODUCTION

DRUG RESISTANCE has been correlated to a broad variety of possible mechanisms including impaired drug influx, enhanced efflux, increased drug degradation, inadequate drug activation, increased levels of target enzymes, development of alternative biochemical pathways bypassing the drug induced block and enhanced repair of drug induced lesions. For reviews see [1–6]. Some of these mechanisms have also been correlated to resistance against alkylating agents. Reduced drug uptake has been described in N-mustard resistant cells [7, 8]. Resistance to cyclophosphamide has in some cases been attributed to enhanced enzymatic inactivation [4, 9]. In other cases, increased amounts of competing nucleophiles, especially thiols, seem to protect essential targets of alkylating agents [4, 10–12]. Finally, differences in the capacity to repair DNA lesions by alkylating agents, especially the removal of DNA-interstrand cross-links or altered rates of removal of mono-adducts, have been discussed as possible mechanisms leading to resistance against alkylating drugs [3, 13–15].

According to a dominating opinion, the formation of DNA-interstrand cross-links is essential for the growth inhibitory effect of bifunctional alkylating agents [16, 17]. On the basis of this hypothesis one would assume that resistance to alkylating agents is caused by either reduced formation (due to decreased uptake or increased inactivation) or increased repair of DNA-interstrand cross-links. This conclusion, however, has been questioned by several reports, indicating similar cross-linking frequencies and identical rates of cross-link repair in cells resistant to alkylating agents [18–21]. Thus, additional mechanisms leading to resistance against alkylating agents have to be postulated.

In previous reports we have demonstrated that alkylating agents at therapeutic concentrations depress the acetylation of core histones [22, 23]. It is shown here that the inhibition of histone

Accepted 8 July 1988.
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acetylation is less expressed in cells resistant to N-mustard than in the corresponding sensitive cell line. The difference in histone acetylation between sensitive and resistant Walker cells is in contrast to identical cross-linking frequencies and repair rates in these cells. The data suggest that—besides DNA cross-linking—inhibition of histone acetylation should be considered as an important alternative mechanism responsible for the cytotoxic activity of alkylating agents. Ehrlich-ascites tumor cells with an acquired resistance to N-mustard also exhibited a smaller inhibition of histone acetylation than the sensitive counterparts which in this case seems to be due to a reduced drug uptake caused by a defective choline carrier.

MATERIALS AND METHODS

Chemicals

N-Methyl-bis(2-chlorethyl)amine/HCl (N-mustard, HN2) was obtained from Aldrich Chemie, Steinheim, F.R.G. Chlorambucil and choline chloride were obtained from Sigma Chemicals, Munich, F.R.G. 4-Sulfonatoethylthiocyclophosphamide (ASTA Z7557) was a gift from ASTA-Werke A.G., Bielefeld, F.R.G. [Methyl-^{14}C]choline chloride (50 mCi/mmol), [2-^{14}C]thymidine (52 mCi/mmol) and [methyl-^{3}H]thymidine (90 Ci/mmol) were products of the Radiochemical Centre, Amersham, U.K. Proteinase K was from Merck A.G., Darmstadt, F.R.G. Tetrapropylammonium hydroxide was obtained from Fluka, Buchs, Switzerland.

Cell culture

Ehrlich-ascites tumor cells were cultured in Dulbecco's modified Eagle's minimal essential medium as described elsewhere [24]. Ehrlich cells resistant to HN2 were obtained as described previously [25]. Walker rat carcinoma cells sensitive and resistant to various antitumor agents were kindly supplied by Dr. J.J. Roberts, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey, U.K. and grown at 36.5°C in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and buffered with 25 mM morpholinopropanesulphonic acid (pH 7.35 at 20°C). Cell multiplication (M) was calculated by $M = T_t - T_0/C_t - C_0 \times 100$, in which T and C correspond to cell numbers of treated (T) or control (C) cells respectively at time 0 (0) and 48 h (t). Cell counting was performed with an electronic counter (Coulter Electronics, Luton, U.K.).

Determination of interstrand cross-links

DNA-interstrand cross-links were determined by the alkaline elution assay as described by Kohn *et al.* [27]. 3.5×10^5 cells labeled with [^{14}C]thymidine were mixed with 4×10^5 internal standard cells labeled with [methyl-^{3}H]thymidine and subjected to 300 rads of X-ray at 0°C. The cells were deposited on polycarbonate filters, 0.8 μm pore size (Nucleopore Corporation, Pleasonton, California, U.S.A.) and lysed with 2% sodium dodecylsulfate (SDS) and 0.05 M tris(hyroxymethyl)aminomethane, pH 9.7. DNA was deproteinized by proteinase K (0.5 mg/ml). The eluting solution (0.1 M tetrapropylammonium hydroxide; 0.02 M EDTA; 0.1% SDS; pH 12.1) was pumped through the filters at a rate of 1.5 ml/h. Fractions were collected at 2 h intervals for a total of 14 h and aliquots counted in a scintillation spectrometer. Cross-link coefficient was determined as $K_c = (1-r_0)/(1-r)^{1/2}-1$, where r and r_0 represent the fractions of DNA of treated (r) and untreated (r_0) cells respectively which remain on the filters after 10 h of elution.

Labeling and isolation of histones from cultured cells

[^{3}H]Acetate (2 Ci/mmol) was added to the medium at a concentration of 40 μCi/ml. At the time points indicated, cells were collected by centrifugation and washed twice in phosphate buffered saline (PBS) (0.14 M NaCl; 2.7 mM KCl; 4.6 mM Na_2HPO_4; 1.7 mM NaH_2PO_4; pH 7.4; 10 mM Na-butyrate; 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM 2-mercaptoethanol). Butyrate, PMSF and 2-mercaptoethanol remained present at all steps during preparation of histones. Cell pellets were frozen by rapid immersion into liquid nitrogen for 2 min and extracted with 0.25 M HCl. Histones were precipitated from the HCl extract by an addition of 20% TCA and dissolved in 0.1 N NaOH. For *in vivo* labeling, Ehrlich-ascites tumor-bearing mice were injected 4 days after tumor transplantation i.p. with 2 mCi [^{3}H]acetate per 30 g animal in 0.2 ml PBS (butyrate-, PMSF and 2-mercaptoethanol-free). After 15 min the animals received 0.5 mg puromycin i.p., and after additional 15 min cells were harvested and nuclei prepared as described by Bloom and Anderson [28]. Nuclei were washed twice in 0.5 M sucrose; 50 mM tris/HCl pH 7.5; 25 mM KCl; 5 mM $MgCl_2$; 10 mM Na-butyrate; 15 mM 2-mercaptoethanol; 0.4 mM PMSF and 0.5% Triton X-100. Histones were extracted with 0.4 N H_2SO_4, precipitated overnight with 5 vol. ethanol at −20°C, dried in a desiccator and finally solubilized in 0.1 N NaOH for counting and protein determination.

Fractionation of chromatin

Chromatin was digested with 100 U (Worthington) micrococcal nuclease per mg DNA at 37°C for 10 min. Incubation was terminated by addition of EGTA (0.1 mM) and shifting the temperature to 0°C. The nuclease digested chromatin is subsequently extracted with NaCl solutions of increasing ionic strength as described by Sanders [29].

RESULTS

1. *Resistance to* N-*mustard in Ehrlich-ascites cells correlates with reduced drug uptake due to defective choline carrier*

Ehrlich-ascites tumor cells resistant to N-mustard were obtained by cultivating the cells in the presence of increasing concentrations of N-mustard. After 6 months of treatment the cells exhibited a 40-fold increased resistance to N-mustard, but were not cross-resistant to cyclophosphamide or chlorambucil (Table 1). After treatment with 1 μM N-mustard, the frequency of DNA-interstrand cross-links was much lower in the resistant compared to the sensitive cells (Fig. 1). About 10 times higher N-mustard concentrations had to be administered to resistant cells in order to reach a similar cross-linking frequency as in sensitive cells. As the repair rates of the DNA–DNA cross-links were identical in both cell lines (Fig. 2) reduced cross-linking frequency has to be due to a depressed cross-link formation.

Total cross-link formation should be a function of intracellular drug concentration (although this is not the only determinant). N-Mustard transport in lymphoid and Walker cells has been shown to be catalyzed by the choline carrier [30]. The same transport system seems to operate in Ehrlich cells as addition of an excess of choline drastically reduces cross-link formation by N-mustard, probably by competitive inhibition of N-mustard transport (Fig. 1). As shown in Fig. 1, choline is much less effective in reducing cross-link formation by N-mustard in resistant Ehrlich cells. This finding indicates that in the resistant cells the amount of N-mustard transported into the cell by the choline carrier is significantly reduced. This conclusion is supported by the data of Fig. 3 demonstrating that the choline carrier in resistant cells exhibits a reduced affinity to choline.

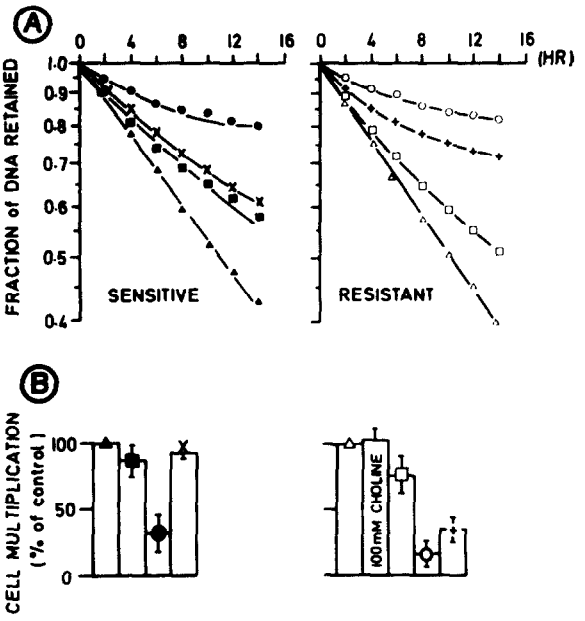

Fig. 1. Formation of DNA-interstrand cross-links (A) and inhibition of cell multiplication (B) after treatment of Ehrlich-ascites tumor cells with N-*mustard (HN2) or a HN2/choline mixture. Cells were treated with the indicated concentrations of HN2 or a HN2/choline mixture where indicated. After* 1 h *cells were separated from the medium, washed once in PBS and either resuspended in fresh medium and kept for an additional* 48 h *to measure drug effects on cell multiplication or treated as described under Materials and Methods for determination of DNA-interstrand cross-links. Sensitive cells:* (▲) *control;* (●) 1 μM *HN2;* (■) 0.1 μM *HN2;* (×) 1 μM *HN2* + 10 μM *choline. Resistant cells:* (△) *control;* (○) 10 μM *HN2;* (□) 1 μM *HN2;* (+) 10 μM *HN2* + 100 μM *choline.*

Table 1. *Antiproliferative effect of alkylating agents on Ehrlich-ascites tumor cells sensitive and resistant to nitrogen mustard*

		Ehrlich sensitive IC_{50} (μM)	Ehrlich resistant IC_{50} (μM)
Drug			
Nitrogen mustard	(48 h)	0.12 ± 0.017*	4.9 ± 0.5
Nitrogen mustard	(1 h)	0.76 ± 0.18	3.23 ± 1.18
Chlorambucil	(48 h)	1.30 ± 0.13	1.6 ± 0.38
Cyclophosphamide†	(48 h)	1.8 ± 0.44	1.2 ± 0.20

*Mean ± S.E.M.

†Sulfonatoethylthiocyclophosphamide (ASTA Z7557).

Ehrlich-ascites cells were grown in suspension culture and adjusted to a cell density of 10^5 per ml. Nitrogen mustard was solubilized in 1 mM HCl, chlorambucil in ethanol and ASTA Z7557 in H_2O and appropriate amounts added to the culture medium. Controls received the solvent only. The maximum final ethanol concentration in the medium was 0.1% and had no effect on cell proliferation. After addition of drugs, cell proliferation was followed during a 48 h period and inhibition of cell growth determined as described under Materials and Methods. Where indicated, exposure to N-mustard was limited to 1 h. In these cases, cells were collected by centrifugation, washed 1 × in 4°C medium and resuspended in fresh medium. Cell numbers were determined after an additional 47 h period. IC_{50} values were determined employing linearized dose–effect plots according to Chou and Talalay [44].

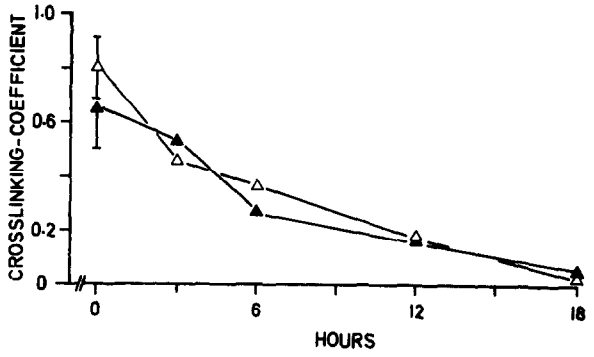

Fig. 2. Repair of DNA interstrand cross-links in N-*mustard (HN2) sensitive and resistant Ehrlich-ascites tumor cells. Cells were treated with* N-*mustard (1 μM sensitive) or (10 μM resistant) cells. After* 1 h *cells were collected by centrifugation, washed and resuspended in fresh medium and cross-links determined at the time points indicated as described under Materials and Methods. (▲) Ehrlich sensitive; (△) Ehrlich resistant.*

2. *Resistance to* N-*mustard in Walker cells is not correlated to altered drug uptake, reduced cross-link formation or increased cross-link repair*

The Walker carcinoma cells which exhibit a reduced sensitivity to N-mustard showed cross-resistance to the cyclophosphamide ASTA Z7557, chlorambucil and adriamycin (Table 2). One hour after treatment with 1 or 10 μM N-mustard, cross-linking frequencies were slightly lower in resistant cells compared to the sensitive parental line. This small difference, however, disappeared after 3 h and the cells not only showed identical cross-linking frequencies, but also the same rates of cross-link removal (Fig. 4). The data indicate that the approx. 25-fold resistance to N-mustard is neither explained

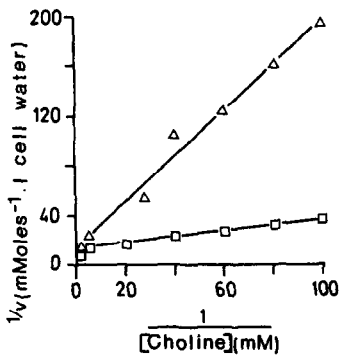

Fig. 3. Choline uptake into N-*mustard sensitive (□) and resistant (△) Ehrlich-ascites cells. Exponentially growing cells were concentrated to a density of* 2×10^6 *cells per* ml *by centrifugation and resuspended in Eagle's minimal essential medium with Eagle's salt buffered with* 20 mM *MOPS at* pH 7.35. 10 μCi/ml *tritiated water was added to the suspension. The concentration of choline in this medium was taken as* 7.5 μM. *After a preincubation period of* 10 *min at* 37°C, 135 μl *aliquots of the cellular suspension were transfered to the test tube with* 150 μl *of the same medium but with different amounts of* [^{14}C]*choline and mixed rapidly.* 10 *min later the cells were separated from the medium by the silicon oil layer method and the amount of choline per liter cell water was calculated as described previously* [26]. *Each point represents the mean of two determinations. Data were plotted by the method of Lineweaver and Burk.*

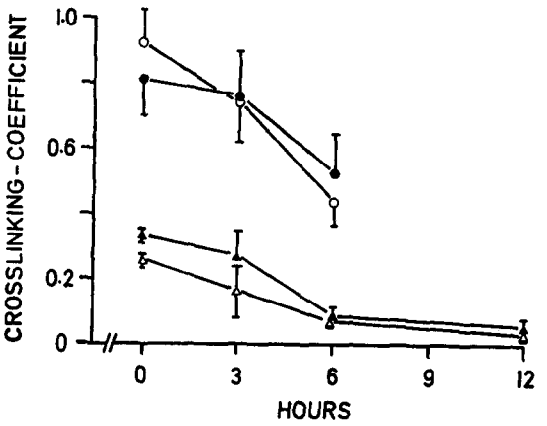

Fig. 4. Repair of DNA-interstrand cross-links in HN2 sensitive and resistant Walker carcinoma cells. Cells were treated with the alkylating agent at the concentrations indicated. After 1 h *cells were collected by centrifugation, washed once in PBS, resuspended in fresh medium and cross-links determined as described under Materials and Methods. Sensitive cells,* 10 μM N-*mustard (●), resistant cells,* 10 μM N-*mustard (○). Sensitive cells,* 1 μM N-*mustard (▲), resistant cells,* 1 μM N-*mustard (△).*

by a depressed drug uptake leading to reduced cross-link formation nor by elevated rates of cross-link repair.

3. N-*mustard reduces histone acetylation*

As shown previously, N-mustard and other alkylating agents depress acetylation of core histones [22, 23]. It was not yet clear, however, whether this effect is caused by an inhibition of the acetyl-transferase reaction or whether it is due to increased deacetylation of histones. Histone deacetylation can be inhibited by butyrate [31–33]. The effect of N-mustard on histone acetylation in Ehrlich-ascites tumor cells in absence or presence of butyrate is shown in Fig. 5. Incorporation of labeled acetate in the presence of the deacetylase inhibitor should predominantly measure acetylation whereas in the absence of the inhibitor the label appearing in the histones is a function of both the acetylating and deacetylating activities. Figure 5A demonstrates that the inhibitory effect of N-mustard is not influenced by Na-butyrate, indicating that the alkylating agent affects the acetyl-transferase reaction and does not increase the rate of deacetylation. That butyrate is effective as a deacetylase inhibitor under the conditions used here was described elsewhere [34]. The conclusion that treatment with the alkylating agent does not lead to an accelerated deacetylation is supported by the pulse-chase experiment shown in Fig. 5B. As can be seen, N-mustard treated and control cells exhibit the same turnover of acetyl groups in histones. This turnover is blocked by Na-butyrate.

4. *Depression of histone acetylation by* N-*mustard is more pronounced in* N-*mustard sensitive than in resistant cells*

The effect of N-mustard on histone acetylation

Table 2. Antiproliferative effect of antitumor agents on Walker carcinoma cells sensitive and resistant to nitrogen mustard

	Walker sensitive IC_{50} (μM)	Walker resistant IC_{50} (μM)
Drug		
Nitrogen mustard	0.054 ± 0.01*	1.31 ± 0.2*
Nitrogen mustard	0.13 ± 0.05†	2.19 ± 0.78†
Chlorambucil	0.246 ± 0.1	6.6 ± 2.2
Cyclophosphamide‡	0.33 ± 0.1	5.01 ± 1.3
Phosphoamide mustard	0.47 ± 0.12	4.56 ± 1.05

*Mean ± S.E.M. (48 h exposure).
†Mean ± S.E.M. (1 h exposure).
‡4-Sulfonatoethylthiocyclophosphamide (ASTA Z7557).
Cells were grown in presence of the alkylating agents for 48 h. IC_{50} values were determined according to Chou and Talalay [44]. For experimental details see footnote to Table 1.

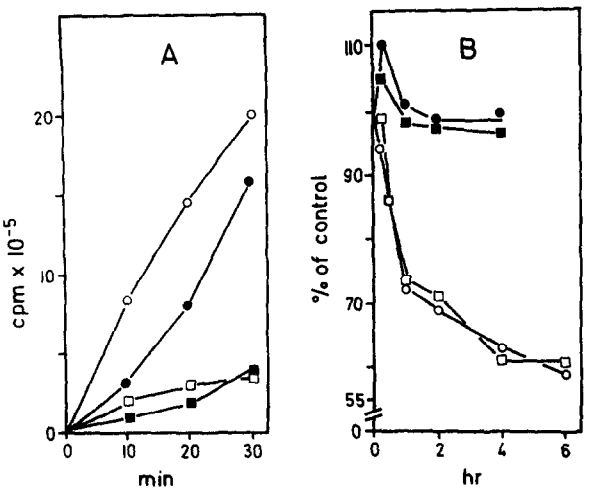

Fig. 5A. Depression of histone acetylation by N-mustard. N-Mustard was added to exponentially growing cells culture at a final concentration of 20 μM. Where indicated, butyrate was administered 3 h after the addition of N-mustard. After an additional hour, cells were pulse labeled with [³H]acetate (40 μCi/ml) and labeled histones prepared as described under Materials and Methods. (○) Controls; (●) controls + butyrate 10 μM; (□) + N-mustard; (■) + N-mustard + butyrate.

Fig. 5B. Effect of N-mustard on the decrease in the specific activity of histones labeled with [³H]acetate. Cells were pulse labeled with [³H]acetate (40 μCi/ml) for 60 min, washed and at 0 time resuspended in fresh medium containing a 120-fold excess of cold acetate and where indicated, 10 μM Na-butyrate. N-Mustard was administered at time 0 at a final concentration of 20 μM. Symbols see Fig. 5A.

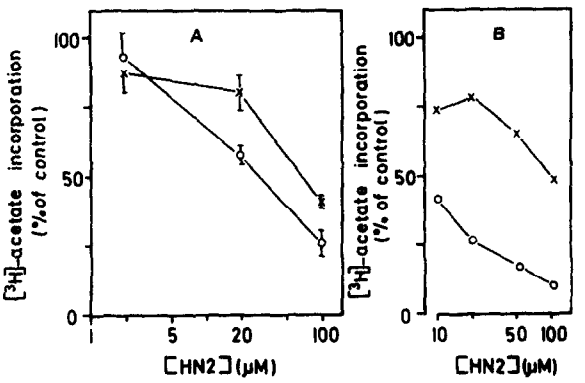

Fig. 6. Inhibition of histone acetylation by N-mustard in sensitive and resistant Walker carcinoma (A) and Ehrlich-ascites cells (B). Cells were treated with the N-mustard concentrations indicated as described in the legend to Fig. 5 and histone acetylation determined as described under Materials and Methods. (○) Sensitive cells; (×) resistant cells.

in N-mustard sensitive and resistant Ehrlich or Walker cells is depicted in Fig. 6. The resistant Ehrlich cells require approx. 10 times higher N-mustard concentrations in order to obtain a similar depression of histone acetylation as in the sensitive parental cells. This corresponds roughly to the ratio between equitoxic concentrations for inhibition of cell multiplication of sensitive and resistant cells after a 1 h exposure to the alkylating agent. The resistant Walker cells require approx. 15-fold higher N-mustard concentrations than the sensitive subline for equitoxic effects on cell proliferation after 1 h treatment. Thus in Walker cells, the difference in histone acetylation between sensitive and resistant cells does not seem to account for the decrease in sensitivity to N-mustard. But, with regard to histone acetylation, the difference between resistant and sensitive Walker cells are definitely more pronounced than differences in DNA cross-linking frequencies on repair rates.

5. *Differential sensitivity of various chromatin fractions to inhibition of histone acetylation by alkylating agents*

The data presented so far suggest that inhibition of histone acetylation is at least as closely correlated to N-mustard sensitivity than DNA-interstrand cross-linking. The inhibition of histone acetylation could be demonstrated at all concentrations of various alkylating agents including cyclophosphamide, which significantly depress cell proliferation [22]. It should be emphasized, however, that in general higher concentrations of alkylating agents are required for a significant inhibition of histone acetylation than for the detection of DNA-interstrand

cross-links. However, the sensitivity of histone acetylation against cyclophosphamide varies if different chromatin fractions are compared. If chromatin is digested with micrococcal nuclease and subsequently extracted with NaCl solutions of increasing ionic strength, the strongest inhibition is seen in the chromatin fraction soluble in 0.1 M NaCl whereas histone acetylation in chromatin soluble in 0.6 M NaCl proved to be unaffected (Fig. 7). The fractions soluble in 0.1 M NaCl have been shown to consist predominantly of mononucleosomes and to be enriched in transcribed sequences [29, 35, 36]. These data suggest that the effect of alkylating agents on histone acetylation depends on the functional state of the chromatin which renders some domains more vulnerable to alkylating agents than others. It is conceivable, therefore, that at low concentrations of alkylating agents, histone acetylation is already blocked at some sensitive functionally important sites whereas overall acetylation is not yet significantly reduced.

DISCUSSION

Cultivation of cells in the presence of increasing concentrations of N-mustard resulted in the selection of cells with a defective choline carrier. The choline carrier is employed by N-mustard for drug uptake in lymphocytes and Walker cells [2]. The data presented here indicate that the same transport system is operative in Ehrlich cells. The defective carrier exhibits a reduced substrate affinity. Therefore, under nonsaturating conditions, less substrate

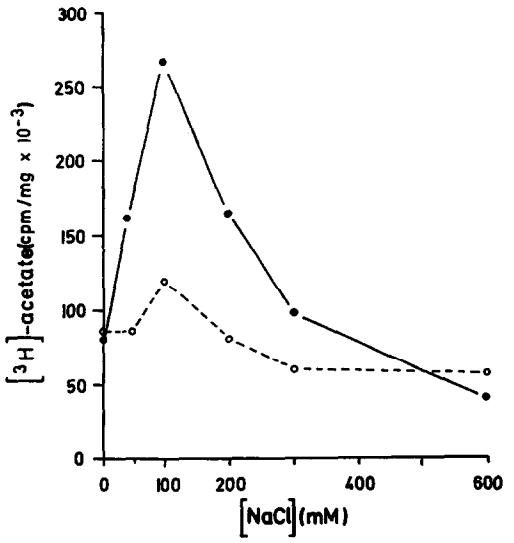

Fig. 7. Effect of cyclophosphamide on histone acetylation in chromatin fractions from Ehrlich-ascites tumor cells. The cyclophosphomide derivative ASTA Z7557 was injected i.p. to yield a final concentration of 10^{-4} moles/kg. *Cells were labeled with* [^{3}H]*acetate in the presence of puromycin and chromatin fractions prepared from micrococcal nuclease digested cells as described under Materials and Methods. The* NaCl *concentrations on the abscissa indicate the salt solutions in which the particular chromatin fraction was solubilized.* (•) *Controls;* (○) + 10^{-4} M *cyclophosphamide.*

(choline or N-mustard) is transported into the cells. The fact that a reduced drug uptake severely reduces drug sensitivity indicates that membrane lesions which have been reported to occur at therapeutic concentrations of alkylating agents [25, 37–40] are less significant for the cytotoxic effect of alkylating agents than intracellular events.

The precise intracellular mechanism by which alkylating agents inhibit cell proliferation is still subject to question. The data presented here do not support the wide spread opinion that formation of DNA-interstrand cross-links represents the essential mechanisms by which bi- or trifunctional alkylating agents inhibit cell multiplication. Sensitivity to N-mustard correlates neither to total cross-linking frequency nor to the capacity to repair these lesions. This is evident from the studies employing sensitive and resistant Walker cells (Fig. 4). A loss of the ability to repair DNA cross-links should of course enhance the sensitivity to cross-linking agents and such a mechanism has been described as being responsible for a collateral sensitivity to chloroethylnitrosourea [41]. But, an increased capacity for repair of DNA lesions does not seem to be the cause of the resistance to N-mustard in the cell systems studied here. Similar conclusions have been drawn by other authors with other systems where a correlation between DNA cross-linking frequency or repair and sensitivity to alkylating agents could not be demonstrated [18–21]. Thus, the cytotoxic activity of bifunctional alkylating agents is either not related to DNA cross-linking at all or in the sensitive cells a few cross-links are formed within critical regions with a frequency below the level of detection of the alkaline elution technique and which for some reason are not accessible to the repair system. At present, however, there is no evidence in support of the latter alternative.

In view of these data, targets other than DNA should be considered. The data shown here indicate that the previously described inhibition of core histone acetylation [22, 23] contributes to the cytotoxic effects of alkylating agents and is correlated to drug sensitivity. As the exact biological role of histone acetylation is still unclear, it is premature to answer the question how an inhibition of histone acetylation leads to reduction of cell multiplication. Preliminary evidence demonstrates a replication-linked histone acetylation preceding the onset of DNA synthesis [42]. Whether replication-linked histone acetylation is particularly sensitive to alkylating agents remains to be shown. Moy and Tew [32] demonstrated reduced phosphorylation of nuclear matrix proteins in N-mustard resistant Walker cells. Whether this also applies to the cells studied here has not been studied. The observation by Moy and Tew [43], however, emphasizes the relevance of nuclear proteins in mechanisms under-

lying resistance to alkylating agents. Several reports stress the importance of intracellular thiols in determining sensitivity to alkylating or platinating agents [4, 10–12]. Altered concentrations of intracellular thiols, however, cannot account for the differences in sensitivity to N-mustard in the system studied here. The fact that both sensitive and resistant Walker cells show equal levels of DNA-interstrand cross-links argues against increased inactivation of N-mustard by elevated concentrations of thiols. In summary, for the tumor cell lines studied here, resistance was found to be correlated to reduced drug uptake and depressed inhibition of histone acetylation.

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