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REDUCTION OF THE TOXICITY OF "RADIOMIMETIC" ALKYLATING AGENTS IN RATS BY THIOL PRETREATMENT PART II. MECHANISM OF PROTECTION

G. CALCUTT* T. A. CONNORS, L. A. ELSON, and W. C. J. ROSS

Chester Beatty Research Institute, Institute of Cancer Research,
Royal Cancer Hospital, Fulham Road, London, S.W.3

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Abstract—Evidence is presented which suggests that cysteine reduces the toxicity and lethality in rats of a number of aromatic nitrogen mustards as a result of an intracellular reaction between the ionized thiol and the carbonium ion formed from the mustard. Correlations have been obtained between the degree of protection afforded by cysteine and (a) the chemical reactivity of the nitrogen mustard and (b) the amount by which cysteine raises the intracellular protein free —SH level of spleen, liver, thymus.

IN A previous paper (part I of this series[1]) it has been shown that cysteine in high doses gave good protection against the toxicity and lethality in rats of two aromatic nitrogen mustards, Merophan (*o*-di-2-chloroethylamino-DL-phenylalanine) and 1-leucylmelphalan ethyl ester hydrochloride. Only slight protection was obtained against eponate, (a *bis*-epoxide), and none against dimethyl Myleran, (a sulphonoxyalkane), or thio-TEPA. It was suggested that the protection afforded by cysteine was related to the chemical reactivity of the nitrogen mustard and involved reaction between the ionized thiol ($R\tilde{S}$) and the carbonium ion ($R_2.N.CH_2CH_2^+$) formed from the mustard. Since thiosulphate, which does not penetrate cells, is extremely reactive towards carbonium ions, but gave no protection against Merophan it was assumed that protection was intracellular. If cysteine protection does involve an intracellular reaction between the ionized thiol and the carbonium ion formed from the mustard then it would be expected that the degree of protection would be dependent on the concentration of thiol in the tissue. Also, the best protection should be obtained against the more reactive nitrogen mustards, since they will form a greater quantity of carbonium ions during the limited period the administered cysteine is still present as such in the cell.[2] The present paper shows that the extent of protection afforded by cysteine is related to the rise in free —SH levels of tissues after cysteine administration and that an association exists between the chemical reactivity of the mustard and the degree of protection.

MATERIALS AND METHODS

Female Wistar rats from an inbred colony, 200–250 gm in weight, and 8 weeks old were used. They were maintained on standard rat cake diet and water *ad lib.* All the

* Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex.

compounds used were administered intraperitoneally in aqueous solution and prepared immediately prior to the injection. Merophan was prepared for injection by dissolving in methanol and adding water to make a 5% solution of methanol in water. Cysteine hydrochloride was dissolved in one equivalent of normal sodium hydroxide and diluted with water. The LD_{50}'s of the nitrogen mustards with and without cysteine pretreatment were determined by the method of Weil[3] using groups of ten animals at four or more dose levels. The hydrolysis rates of the mustards, which are a measure of their chemical reactivity, were determined by the method of Ross.[4] Tissue sulphydryl estimations were by the method described by Calcutt and Doxey,[5] and Calcutt, Doxey and Coates.[6]

RESULTS

It had previously been shown (Table 1) that, for the best reduction in toxicity, the optimum time for administration of cysteine was no later than 30 min before administration of the mustard. It was not practicable to give the mustard less than 30 min after the thiol, since at 15 min a small quantity of cysteine remains in the peritoneum and as both mustard and cysteine are given intraperitoneally there was a possibility of local reaction in the peritoneum. At 30 min although there was a large quantity of fluid in the peritoneum all the cysteine appeared to have been absorbed.

TABLE 1. REDUCTION OF THE TOXICITY OF MEROPHAN (9 mg/kg, i.p.) BY L-CYSTEINE HYDROCHLORIDE (1000 mg/kg, i.p.)

Pretreatment	Time of Administration of cysteine	Number of deaths	% of animals protected
Cysteine	2 hr before	10/10	0
Cysteine	1 hr before	4/10	60
Cysteine	30 min before	0/10	100
None	—	10/10	0
Cysteine	30 min after	10/10	0

TABLE 2. PROTECTION AGAINST MEROPHAN USING VARIOUS DOSES OF L-CYSTEINE HYDROCHLORIDE (GIVEN 30 MIN BEFOREHAND)

Pretreatment dosage of cysteine (mg/kg)	LD_{50} Merophan (mg/kg)	Dose reduction factor
None	3·67	
62·5	3·99	1
125	3·99	1
250	6·50	1·8
500	11·02	3·0
1000	15·24	4·2

Table 2 shows the protection afforded against the lethality of Merophan using doses of cysteine ranging from 62·5 mg/kg to 1000 mg/kg given 30 min before the mustard. From Fig. 1 which shows the effect of doses of cysteine from 125 mg/kg to 1000 mg/kg in raising the protein free —SH level of the spleen, it can be seen that there is a direct correlation between the extent of whole body protection and the rise of

free —SH in the spleen. 1000 mg/kg of cysteine increased the free —SH level to 220 per cent of the normal value and this dose gave a dose reduction factor for Merophan of 4·2, while 250 mg/kg of cysteine which increases the free —SH to about 140 per cent of the normal value gave a dose reduction factor of 1·8. 125 mg/kg of cysteine and lower doses which gave no significant protection had no effect on the free —SH levels of the spleen. From Fig. 1 it can be seen that the highest concentration of free —SH occurs between 30–45 min after the injection of cysteine. We have already shown (Table 1) that the optimum time for cysteine injection is about 30 min before the

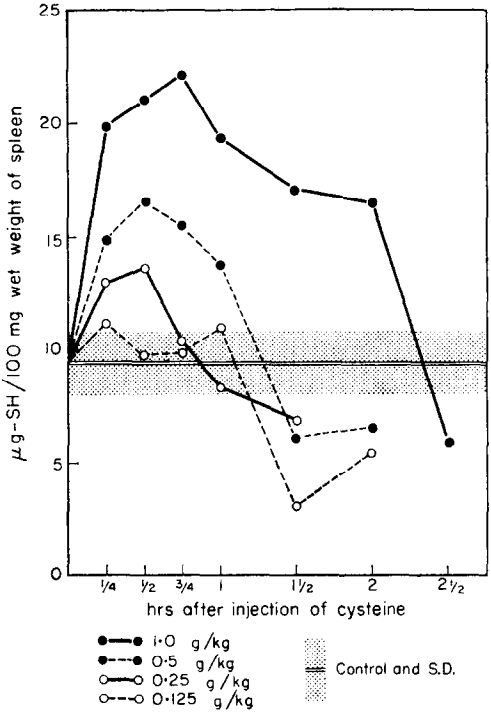

FIG. 1. Effect of injected cysteine on the -SH levels of rat spleen.

TABLE 3. PROTECTION OBTAINED AGAINST VARIOUS AROMATIC NITROGEN MUSTARDS USING L-CYSTEINE HYDROCHLORIDE (1000 mg/kg i.p. 30 MIN BEFOREHAND)

Compound	LD_{50} (mg/kg)	LD_{50} (mg/kg) after cysteine pretreatment	Dose reduction factor	Hydrolysis rate (%)
Merophan	3·67	15·24	4·2	38
Melphalan ethyl ester hydrochloride*	8·7	15·4	1·8	24
1-Leucylmelphalan ethyl ester hydrochloride*	13·4	31·8	2·5	22
m-Phenylglycine mustard	24·4	55·1	2·3	13
p-Benzoic acid mustard sodium salt	109·5	131·5	1·2	12

* Corrected for the effect of chloride.[18]

mustard. The rise in free —SH levels of liver and thymus after cysteine administration were essentially the same as for spleen.

Table 3 gives the dose reduction factors (using cysteine 1000 mg/kg, 30 min before hand) obtained for a range of aromatic nitrogen mustards of different chemical reactivity. The value obtained for the hydrolysis rate is only an indication of the relative order of reactivity these compounds might be expected to have *in vivo* and it is of interest that the more chemically reactive mustards are also the more toxic. An association is apparent between the reactivity of the mustard and the extent of protection.

DISCUSSION

Patt *et al.*[7] have shown that the degree of protection afforded by cysteine against radiation induced toxicity increases linearly with the logarithm of the cysteine dose. Cysteine protection against Merophan increases linearly with the logarithm of the cysteine dose, when doses of cysteine of 125 mg/kg or greater are used. The extent of radiation protection obtained with thiols has been shown in some cases to be dependent on the dose rate[8, 9] and this also would be analogous to our findings that protection against mustards is dependent on their chemical reactivity.

Our results indicate that cysteine protection can be accounted for by a reaction between the ionized thiol and the mustard, and is in agreement with the work of Salerno and Friedell[11] who concluded that cysteine protected against HN2 by reacting with the ethylene-imonium ion formed from the mustard. However, the efficacy of all thiols in protecting against radiation induced lethality or mustard lethality cannot be determined entirely by their ability to increase the free —SH levels of tissues. It is well known that thio-glycollate, for instance, can increase the sulphydryl content of tissue but affords no protection against either radiation induced toxicity[11] or Merophan.[1]

Also cysteamine, which gives only poor protection against Merophan lethality when compared to cysteine, is as effective as cysteine against radiation induced toxicity. The increases in free —SH levels of spleen, liver and thymus after cysteamine are similar to those obtainable with cysteine. In the case of cysteamine protection against the toxicity of HN2 it would appear that the mechanism of protection depends not on a reaction between the thiol and the mustard, but on the formation by cysteamine of mixed disulphides, with essential thiol groups, preventing their alkylation by the mustard.[12, 13] The nature of the thiol contributing to the increased —SH level and the distribution in the cell would seem to be important factors or perhaps the inter-relationship between protein bound and protein free —SH and disulphide formation after administration of the thiol must be taken into consideration. There is also a possibility that a rise in free —SH in a particular organ is responsible for the reduction in lethality.

Practical importance of the finding that cysteine can reduce the lethality of some mustards lies in the possibility of protecting the host during localized chemotherapeutic treatment by increasing the effective thiol levels in regions to be spared from the cytotoxic action of the alkylating agents. This could be done by systemic administration of thiols to the host in cases where localized perfusion techniques are used, and by using thiol–glucose combinations to achieve a selective concentration of the reactive detoxifying form ($R\bar{S}$) in normal cells as compared with cancer cells.[1]

Schmidt,[14] Skipper and Schabel,[15] Hirono,[16] and Oboshi[17] have established an

interesting cross resistance in experimental animal tumours between a range of alkylating agents, including mustards, ethylene-imines and sulphonoxyalkanes. This cross resistance suggests that either the cells had acquired the ability to reverse the process of alkylation or they were able to inactivate agents before they could reach target sites. One way in which the latter effect could be achieved is suggested by the present study, that is, by an increase in the thiol group concentration in resistant cells.

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