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REDUCTION OF THE TOXICITY OF "RADIOMIMETIC" ALKYLATING AGENTS BY THIOL PRETREATMENT—VI

THE MECHANISM OF PROTECTION BY CYSTEINE

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Abstract—The solid Yoshida sarcoma in rats can be protected approximately twofold against the cytotoxicity of the alkylating agent melphalan if the animals are pretreated with cysteine. Using labelled melphalan it has been shown that in cysteine pretreated rats the uptake of the alkylating agent by the tumour is not affected but reaction with the nucleic acids and nuclear protein of the tumour is much reduced. *In vitro* experiments have shown that cysteine can prevent the reaction between melphalan and nucleic acids to the same extent as that occurring *in vivo*. A correlation has been shown between the reactivity of thiols *in vitro* towards melphalan and their ability to protect whole animals.

INTRODUCTION

THE USE of the term "radiomimetic" to describe the pharmacology of the alkylating agents has proved confusing since there is no doubt that the similarities observed between the effects of X-irradiation and alkylating agents in animals are largely superficial. Although toxicity arising from both X-irradiation and alkylating agents may be reduced by pretreating animals with certain thiols, a thiol which protects well against X-irradiation does not necessarily protect against alkylating agent toxicity.[1] Theories put forward to explain the way in which thiols protect animals against X-rays have often been extended to include protection against alkylating agents,[2] but there is no evidence for the assumption that the protective mechanism is the same in both cases. Since thiols are extremely reactive towards alkylating agents *in vitro* the simplest explanation of their protective effect *in vivo* is that they react directly with the alkylating agent so preventing the agent damaging essential sites in the cell. In support of a direct reaction between the thiol and alkylating agent as a mechanism of protection ("competitive removal") it has been shown that thiols which react most readily with alkylating agents are the best protectors, provided high doses are tolerated by the animal.[1] The degree of protection is related to the non-protein thiol level of tissues and protection only occurs when this level is high.[3] It has also been shown that in rats pretreated with cysteine and well protected, the alkylating agent HN2 is excreted more rapidly and reaction products of HN2 and cysteine have been identified.[4] One objection to competitive removal being the mechanism of protection was that although an increased protein free thiol level of tissues had been demonstrated following the administration of a protective thiol, cysteine,[3] it had not been shown

that this increase was due to cysteine itself. This led to speculation that the administered thiols *per se* were not protective but they caused release of another thiol which was the true protector.[5] It has recently been shown, however, that after rats have been injected with cysteine the thiol that accumulates in all tissues is cysteine itself.[6] Up to this time there has been no direct evidence that the protective thiols actually prevent the alkylating agents from reacting with essential sites, as would be expected if protection were by competitive removal. There is good evidence that alkylating agents cause their toxicity by direct alkylation of DNA[7,8] and if thiols protect by reacting with alkylating agents they should restrict this alkylation.

In this paper it is shown that thiols can restrict alkylation of DNA by the nitrogen mustard Melphalan (*p*-di-2-chlorethylamino-L-phenylalanine). Two types of experiment have shown this effect.

a. In model experiments the extent of alkylation of DNA by physiological concentrations of H^3-melphalan has been measured. This reaction has then been carried out in the presence of cysteine at similar concentrations to those occurring in tissues of animals following injection of protective amounts.

b. In whole animals a particular tissue (Yoshida sarcoma) has been shown to be protected about two-fold against the toxicity of Melphalan if the animals have been pretreated with cysteine hydrochloride (1 g/kg). The extent of alkylation of the DNA of the tumour has been measured after injection of H^3-melphalan and this has been compared with the aklylation of DNA seen in animals given the same dose of melphalan but pretreated with cysteine.

MATERIALS AND METHODS

Pharmacology

Female Chester Beatty rats, 200–250 g in weight and 8-weeks old were used in all experiments and maintained on rat cake and water *ad libitum*. Yoshida sarcomas sensitive to nitrogen mustards were transplanted in the inguinal region and the effect of melphalan on the 7-day-old tumour was measured as previously described.[9] The dose of melphalan to cause 90 per cent tumour inhibition (ID_{90}) was estimated in animals given melphalan alone, or pretreated with cysteine hydrochloride (1 g/kg, intraperitoneally in neutral solution) 30 min beforehand. The degree of protection given to the tumour by cysteine was calculated from the ratio:

$$\frac{ID_{90} \text{ of melphalan in cysteine pretreated animals}}{ID_{90} \text{ in animals given melphalan alone.}}$$

The cysteine level in the tumour at different times after treatment with cysteine hydrochloride (1 g/kg) was measured using a method previously described for estimation of cysteine in the presence of glutathione.[6]

H^3-Melphalan

4-N, N-di-(2′-chloroethyl)amino-L-phenylalanine-2-H^3 was prepared by Wade and Murthy[10] with a specific activity of 30 mc/m-mole. A solution of the H^3-melphalan in ethanolic-hydrochloric acid was chromatographed by ascending chromatography on Whatman No. 1 paper in aqueous-ethanol (3:7) and the paper scanned using a Nuclear Chicago Actigraph III strip scanner. This showed 92 per cent of the activity to have the same R_f (0·86) as commercial unlabelled melphalan which was run as a

marker and stained with ninhydrin. The remainder of the activity in the H^3-melphalan was associated with ninhydrin positive impurities also appearing in unlabelled melphalan (7 per cent at R_f, 0·53; 1 per cent at R_f, 0·07) After 21 days a solution of H^3-melphalan in 40% propylene glycol-phosphate buffer was negative to Epstein reagent, which is specific for alkylating function.[11] Since the solution contained no alkylating function it was considered reasonable to use it as 'hydrolysed melphalan" in subsequent experiments.

Scintillation counting

In all the experiments below samples were assayed for radioactivity in an ethanol-dioxan-toluene based scintillation fluid containing POP, POPOP, and napthalene using a Packard Tricarb scintillation counter (Model 3314) with automatic Ra^{226} standardization. Counting efficiency in quenched samples was obtained from a calibration curve of Ra^{226} counts against counting efficiency for tritium in standard samples of H^3-hexadecane quenched by addition of different volumes of chloroform. In low activity samples counts above 70 cpm (background 45 cpm) were accepted with a counting error of less than 1 per cent.

DNA and RNA samples were dissolved in 0·5N perchloric acid by heating at 50°, the solution centrifuged and 0·1–0·25 ml aliquots assayed for radioactivity. The accurate concentration of the assayed solutions was measured by optical density measurements at 260 mμ. DNA (Salmon testes, BDH) and RNA (Yeast, Sigma) were used for calibration curves and also in the *in vitro* experiments described below.

Proteins isolated by phenol extraction were dissolved in 0·1N NaOH by heating at 50° and 0·1–0·3 ml aliquots assayed for radioactivity. Urine samples were assayed by addition of 0·1 ml directly to the scintillation fluid.

Urinary excretion

A group of rats were treated with 1 g/kg cysteine hydrochloride followed 30 min later by 2 mg/kg H^3-melphalan. A control group received 10 ml/kg of 4% sodium chloride solution 30 min prior to the same dose of melphalan. Urine was collected into a freezing mixture from individual animals and assayed for radioactivity as described above.

Isolation of nucleic acids and proteins

The extraction and purification procedures described are based on those described by Kirby.[12,13] Two groups of rats bearing bilateral tumours were treated with H^3-melphalan (4 mg/kg) intraperitoneally, one group having been treated with cysteine hydrochloride (1 g/kg) 30 min earlier. One hour after malphalan treatment the animals were killed, the tumours removed and 5 g immediately homogenized in an aqueous solution of napthalene-1:5-disulphonic acid sodium salt (0·015M, 60 ml) for 45 sec with a Waring homogenizer. The homogenate was added to phenol reagent (60 ml) and shaken on a Rollamix shaker for 20 min at room temperature. The phenol reagent consisted of phenol (500 g), water (55 ml), *m*-cresol (70 ml) and 8-hydroxyquinoline (500 mg). After shaking the mixture was centrifuged at 5° for 40 min at 3500 *g*. The upper aqueous layer (containing RNA) and the lower phenol layer (containing cytoplasmic protein) were removed and the remaining interface

centrifuged at 0° for 10 min at 20,000 *g*. The packed interface was removed, suspended in 6% *p*-aminosalycylic acid solution (60 ml) by shaking, phenol reagent (60 ml) added and the mixture shaken for 20 min as before. Centrifuging again gave an upper aqueous layer (containing DNA) and a lower phenol layer (containing nuclear protein). DNA was precipitated by addition of 1 vol. of 2-ethoxyethanol, RNA by 2 vol. of ethanol and both protein fractions by 15 vol. of methanol-ether (1:2).

The precipitated DNA was removed on a glass rod, dissolved in a minimal volume of 2% sodium chloride solution and treated with RNA-ase at 2° for 18 hr. Solid sodium chloride was then added to increase the concentration to 1 M and the solution extracted with 0·5 vol. of phenol reagent by shaking for 20 min. After centrifugation the aqueous layer was removed and then centrifuged at 40,000 *g* at 0° for 1 hr. DNA was precipitated from the clear supernatant by addition of 0·75 vol. of 2-ethoxyethanol, washed 3 times in 70% ethanol and vacuum dessicated.

The RNA was washed twice in 3M sodium acetate (adjusted to pH 6·5 with acetic acid) by shaking for 2–3 min at 0–5°. It was then successively washed in 75% ethanol containing 1% sodium acetate, 70% ethanol and absolute ethanol and vacuum dessicated. The proteins were triturated twice with methanol:ether (1:2), twice with ether and then vacuum dessicated. Five grammes of tumour yielded approximately 6 mg DNA containing less than 5% RNA and less than 2% protein and about 30 mg RNA containing less than 5% DNA and less than 2% protein.

In some experiments larger DNA yields were required in order that low counts could be estimated accurately. Homogenization of 10 g of tumour directly in 6% *p*-aminosalycylic acid gave after phenol extraction an aqueous layer which yielded about 25 mg DNA after precipitation and purification in the usual way.

Radioactivity of whole tumour homogenates was assayed by homogenizing 1 g wet weight of tumour in 3 ml of distilled water. Two millilitres was evaporated to dryness in a weighed dish for estimation of dry weight. A further 0·5 ml aliquot was dissolved in 25% aqueous tetraethylammonium hydroxide and assayed for radioactivity as described above.

In vitro *experiments with H^3-melphalan*

The relative effectiveness of various thiols in preventing reaction between H^3-melphalan and DNA *in vitro* was investigated using the following reaction mixture. A thiol solution (1 m molar, 10 ml) was added to an equal volume of DNA solution (1 mg/ml in 0·01 M phosphate buffer pH 7·0) followed by 0·1 ml of a solution of H^3-melphalan (1 mg/ml, 3 mc/m-mole) in 0·04% hydrochloric acid in ethanol. The common ion effect made it necessary to have all reaction mixtures at the same chloride concentration. The reagents sodium thiosulphate, thiourea, homocysteine, glutathione and 2,3-dimercaptopropanal (BAL) were dissolved in 1 m-molar sodium chloride solution, cysteine hydrochloride and cysteamine hydrochloride in 1 m-molar sodium hydroxide solution and β-S-2-ethylaminoisothiouronium dihydrobromide (AET) in 2-m-molar sodium hydroxide. The latter solution was allowed to stand for 5 min before use so that complete rearrangement to mercaptoethyl guanidine could occur. Dihydrolipoic acid (Sigma) was dissolved in an equivalent of sodium hydroxide solution and diluted to the required concentration with 1 m-molar sodium chloride solution. Control reaction mixtures contained 1 m-molar sodium chloride (or 2 m-molar sodium bromide) solution in place of the thiol solution. In experiments at

thiol concentrations other than 0·5 m-molar, controls contained the equivalent concentration of sodium chloride. The binding of hydrolysed melphalan was measured similarly.

The reaction mixture was allowed to stand at room temperature for 30 min, solid sodium chloride then added to increase the concentration to 1 molar and the DNA then precipitated by addition of 1 vol. of 2-ethoxyethanol. The DNA was dissolved in 2% sodium chloride solution and reprecipitated by the same procedure. The precipitate was washed in 70% ethanol and vacuum dessicated; 70–80 per cent of the initial DNA was recovered. The specific activity of the DNA was expressed as per cent of a control at the same sodium chloride (or sodium bromide) concentration.

The reaction conditions for the experiments with RNA were identical to those for DNA. After the 30 min reaction period the RNA solution was made 1 molar to sodium chloride and the RNA precipitated by addition of 2 vol. of ethanol. The precipitate was redissolved in 0·01 M phosphate buffer, pH 7·0, reprecipitated as before, washed in 70% ethanol, ethanol and ether, and vacuum dessicated.

A solution of horse serum proteins in 0·01 M phosphate buffer, pH 7·0, was obtained from commercial horse serum (Burroughs-Wellcome, No. 2) by ammonium sulphate precipitation and dialysis. Reaction conditions were similar to those for DNA and RNA, with a final protein concentration of 0·5 mg/ml. A bovine serum albumin calibration curve was used for estimation of protein solution concentration by optical density at 280 mμ. After reaction with H^3-melphalan the protein was precipitated by the addition of saturated ammonium sulphate solution (1·5 vol.). Phenol reagent was added to the wet precipitate, the mixture shaken and then centrifuged. The phenol layer was removed and the protein precipitated by addition of 15 vol. of methanol:ether (1:2). The precipitate was washed twice in methanol:ether, once in ether and assayed for radioactivity as described above.

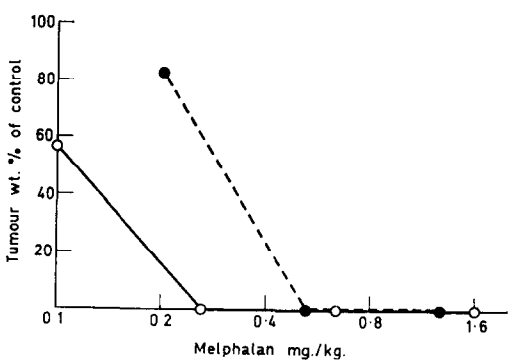

FIG. 1. The effect of melphalan on the growth of the solid Yoshida sarcoma. ●---● animals pretreated with cysteine hydrochloride (1 g/kg; i.p.) 30 min prior to the melphalan. ○—○ animals receiving melphalan only.

RESULTS

The effect of different doses of melphalan on the growth of the solid Yoshida sarcoma is shown in Fig. 1. In animals pretreated with cysteine the cytotoxic effect of melphalan is reduced. The dose reduction factor obtained from the ratio of the ID_{90} doses is 2·3 and is slightly greater than that for the whole animal. After intraperitoneal injection of cysteine hydrochloride (1 g/kg), cysteine is detectable in the

tumour within 5 min and reaches a peak concentration of about 6 μmoles/g wet weight in 30 min (Fig. 2) and the level is still high 90 min after injection. In rats given H^3-melphalan (4 or 8 mg/kg, i.p.) radioactivity was associated with DNA, RNA and

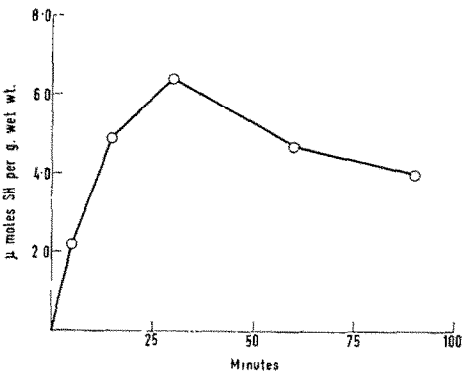

FIG. 2. The cysteine concentration of the Yoshida sarcoma following administration of cysteine hydrochloride (1 g/kg; i.p.).

proteins 1 hr after injection (Table 1). When the animals were treated with cysteine prior to the injection of melphalan the radioactivity associated with all fractions except cytoplasmic protein was much reduced. Table 1 also shows the radioactivity of these fractions after administration of hydrolysed melphalan (4 mg/kg). Significant

TABLE 1. REACTION OF H^3-MELPHALAN WITH CELL FRACTIONS OF THE YOSHIDA SARCOMA *in vivo*

Cell fraction	Control Specific activity (counts/min/mg) after H^3-melphalan at dose stated (Mean ± S.E.)		Cysteine pretreated Specific activity (counts/min/mg) with cysteine treatment before H^3-melphalan (Mean ± S.E.)		Hydrolysed melphalan treated Specific activity (Mean ± S.E.)
	4 mg/kg	8 mg/kg	4 mg/kg	8 mg/kg	4 mg/kg
DNA	102 ± 4	241 ± 17	25 ± 3	40 ± 4	<5
RNA	174 ± 9	366 ± 19	40 ± 4	70 ± 3	<5
Nuclear protein	165 ± 22	312 ± 1	62 ± 12	64 ± 5	19 ± 4
Cytoplasmic protein	283 ± 24	550 ± 30	281 ± 27	298 ± 19	334 ± 25

counts were only obtained on cytoplasmic and nuclear protein. Cysteine pretreatment did not affect the uptake of melphalan by the tumour. One hour after intraperitoneal injection of 4 mg/kg melphalan the activity of tumours of animals pretreated with cysteine was 1600 cpm per mg dry weight and of animals receiving melphalan alone, 1720 cpm per mg dry weight.

The results of the *in vitro* experiments are shown in Table 2. The conditions for the experiments have been selected to approximate as closely as possible to those occurring *in vivo*. Thus a concentration of 5γ/ml of melphalan has been used and this would be the expected maximum concentration of melphalan in the tumour assuming

uniform distribution in animals receiving 5 mg/kg of melphalan by intraperitoneal injection. One of the cysteine concentrations employed has been 5 m-molar. Figure 2 shows that the concentration of cysteine in the tumour at the time the melphalan is given is 6·5 μmoles/g wet wt. (which is equivalent to 6·5 m-molar) and the concentration is approximately 5 m-molar for at least 90 min which is the half life of the alkylating agent. It is apparent from Table 2 that the reaction between DNA and melphalan

TABLE 2. THE EFFECT OF VARIOUS THIOLS IN REDUCING THE ALKYLATION OF DNA BY H^3-MELPHALAN *in vitro*

Compound	Concentration (m-molar)	Specific activity of DNA (% control)
Sodium thiosulphate	0·0005	99·8
	0·005	88·9
	0·05	4·3
	0·5	0·6
L-Cysteine	0·05	98·5
	0·5	57·5
	5·0	21·0
	50·0	9·8
Glutathione	0·5	71·0
Dihydrolipoic acid	0·5	45·0
DL-Homocysteine	0·5	78·5
2,3-Dimercapto-propanol	0·5	41·5
AET	0·5	70·0
Thiourea	0·5	72·0
Cysteamine	0·5	76·0
Sodium chloride*	0·5	100·0
	5·0	80·0
	50·0	21·0

* Effect of sodium chloride concentration—as per cent of 0·5 m-molar control.

TABLE 3. THE EFFECT OF CYSTEINE ON ALKYLATION OF DNA AND RNA BY H^3-MELPHALAN *in vitro*

Concentration of cysteine (m-molar)	Specific activity of DNA (% control)	Specific activity of RNA (% control)
0·05	98·5	—
0·5	57·5	49·0
5·0	21·0	19·0
50·0	9·8	7·7

can be reduced by thiosulphate and a range of thiols at concentrations of 5 m-molar and less. Cysteine at 5 m-molar can also reduce the reaction of melphalan with RNA (Table 3). Hydrolysed melphalan at the same concentration as melphalan showed no binding to DNA or RNA but a considerable binding to protein (Table 4).

Assay of the radioactivity of the urine of rats given 2 mg/kg tritiated melphalan showed that less than 10 per cent of the administered dose was excreted by this route in 96 hr. There was no difference in rate or amount of label excreted if the animals were pretreated with cysteine (Table 5).

DISCUSSION

Although this paper is concerned with the elucidation of the mechanism by which thiols protect against the toxicity of alkylating agents, we should perhaps first mention two points arising from the results which may be relevant to the mechanism of action of alkylating agents. Firstly, it seems that, *in vivo*, much of the radioactivity associated with cytoplasmic protein represented absorbed or incorporated rather than alkylated material, since counts on this fraction were high after administration of hydrolysed melphalan (Table 1). This was confirmed in the *in vitro* experiments where hydrolysed melphalan showed no reaction with DNA or RNA but was associated with protein

TABLE 4. *In vitro* REACTION OF HYDROLYSED MELPHALAN WITH DNA, RNA AND PROTEIN

	Hydrolysed melphalan 5γ/ml
DNA	2
RNA	5
Protein	100

The results were expressed as per cent of the reaction obtained with H^3-melphalan.

TABLE 5. URINARY EXCRETION OF TRITIUM BY RATS AFTER TREATMENT WITH H^3-MELPHALAN (2 mg/kg)

Time	Control (% dose excreted)	Cysteine pretreated (% dose excreted)
0–24 hr	5·76	5·70
24–48 hr	0·43	0·47
48–96 hr	0·03	0·04

TABLE 6. RELATIONSHIP BETWEEN *in vivo* PROTECTION TO WHOLE ANIMALS AGAINST THE TOXICITY OF MEROPHAN AND *in vitro* "PROTECTION" TO DNA

Compound	Maximum tolerated dose (m-moles SH/kg)	Dose reduction factor	Reactivity* towards melphalan
Cysteine	6·3	4·2	42·5
Thiourea	15·8	3·0	28·0
AET	0·9	2·0	30·0
Glutathione	3·3	1·0	29·0
2,3-Dimercapto-propanol	0·4	1·0	58·5
Cysteamine	0·8	1·0	24·0

* Per cent reduction in the reaction of H^3-melphalan with DNA *in vitro* in the presence of the thiol at a concentration of 0·5 m-molar—see Table 2.

as much as melphalan itself (Table 4). It was also found that the sodium chloride concentration could considerably influence the reaction between melphalan and DNA (Table 2). A concentration of 50 m-molar sodium chloride was as effective in reducing DNA alkylation by the common ion effect as many of the thiols tested (Table 6). Since the plasma concentration is of the order of 100 m-molar with respect

to chloride very little reaction with alkylating agents may occur with extracellular constituents. Generally, intracellular chloride concentrations range 5–20 m-molar and some influence on the rate of reaction of melphalan would still be expected. The intracellular chloride concentration of individual tissues may well contribute to the sensitivity of particular tissues to alkylating agents, particularly if the agent is fairly rapidly removed from the body.

The protective effect of cysteine against the toxicity of melphalan can clearly be mediated by competitive removal. In the *in vitro* experiments a concentration of 5 m-molar cysteine allows only 20 per cent of the reaction that normally takes place between melphalan and DNA to occur (Table 2). There is a similar concentration of cysteine in the tumour during the period that the administered alkylating agent is acting. One might therefore expect that by competitive removal *in vivo*, the reaction between melphalan and DNA could be reduced by as much as 80 per cent. Cysteine pretreatment does in fact reduce the reaction of melphalan with nucleic acids and nuclear protein by about 75 per cent (Table 1). Cysteine pretreatment does not affect the uptake of melphalan by the tumour since the radioactivity of the cytoplasmic protein fractions is not reduced by pretreatment, nor is there any reduction in the total radioactivity in whole tumour homogenates.

No greater excretion of label occurred in the urine of animals pretreated with cysteine (Table 5), in contrast to two earlier reports where increased excretion of labelled HN2 was observed in the urine of animals which had been pretreated with either cysteine[4] or cysteamine.[14] However, since the binding of melphalan to cytoplasmic protein is unaltered by cysteine pretreatment, the total decrease in bound material is relatively small and might not be detectable as an increased level of excretion in the urine where the radioactivity recovered in 96 hr only represents 5 per cent of the total administered dose.

It appears that, if a thiol is to be protective to whole animals it must possess two properties. Firstly, it must be well tolerated so that large doses may be given and a high cellular concentration achieved. Secondly, it must be very reactive towards alkylating agents as measured *in vitro*. This is demonstrated in Table 6. In this table is shown the protection given to rats against the melphalan analogue merophan after pretreatment with various thiols given at the maximum tolerated dose. Also shown is the maximum tolerated dose of each thiol expressed in m-moles of SH which should be a measure of the tissue concentration it attains, and the reactivity of each thiol to melphalan as measured by its ability to restrict alkylation of DNA by melphalan *in vitro*. The best protector, cysteine, is both very reactive towards melphalan and has a high maximum tolerated dose. Although thiourea can be given at higher dose levels than cysteine, and presumably brings about a higher concentration of intracellular SH (as isothiourea) it is less reactive towards melphalan and in fact is not as good a protector as cysteine. Glutathione can also be given at high dose levels but it is not protective. Its reactivity to melphalan is low and in the case of this compound there is evidence that it does not penetrate cells as rapidly as cysteine.[6] Conversely, 2,3-dimercaptopropanol reacts more readily with melphalan than cysteine yet it affords no protection *in vivo* since it is so toxic that only small dose levels may be administered. AET is atypical in that it has a relatively high toxicity and a low reactivity towards melphalan, yet is a good protector. There has been a report that AET accumulates selectively in bone marrow[15] and this is confirmed by the finding that this is one of the

few thiols that improves the anti-tumour action of nitrogen mustards by protecting the host more than the tumour.[16,17] If AET is in fact selectively concentrated in body tissues sensitive to the cytotoxic action of the nitrogen mustards, it may be a better protector than would have been predicted from the dose administered. Cysteamine with a toxicity and a reactivity towards melphalan of the same order as AET is unprotective, possibly due to a different distribution in tissues. In general, the protective effect of a thiol may be measured by its reactivity towards the alkylating agent and the level of the intracellular concentration of the thiol achieved during the time the alkylating agent is present in tissues. These two criteria would be expected to be of importance if the mechanism of protection depended on competitive removal. While the maximum tolerated dose of the thiol is a good indication of the intracellular concentration it may achieve, this is not necessarily always the case as has been indicated for glutathione and AET. Thiosulphate should be a very good protective agent, since it is well tolerated (maximum tolerated dose 2 g/kg) and is extremely reactive towards melphalan (Table 2). This compound in fact protects very effectively against the toxicity of HN2 in rats.[18] Its failure to protect against aromatic nitrogen mustards such as melphalan, which alkylate by an SN1 mechanism, has been accounted for by its inability to penetrate cells.[18] A compound capable of entering cells and releasing thiosulphate intracellularly would, if non-toxic, be expected to be an extremely good protector. The high affinity of dihydrolipoic acid for reaction with melphalan (Table 2) was of particular interest since it is a naturally occurring thiol and is required for both carbohydrate metabolism and DNA synthesis.[19,20] If this compound is present in its reactive form in cells, its depletion by reaction with alkylating agents may be significant to the toxicity of alkylating agents.

The alternative hypothesis for the mechanism of protection by thiols is that the thiol protects essential SH groups from alkylation by mixed disulphide formation.[14] Such a mechanism could explain the reduction in nuclear protein alkylation, noted above, in cysteine pretreated animals. It cannot, however, explain restricted alkylation of nucleic acids, the site at which the alkylating agents are thought to exert their cytotoxic effect.

In summary it has been shown that protective thiols are those which can effectively reduce alkylation of DNA *in vitro* by melphalan, and also have high maximum tolerated doses *in vivo*. The *in vivo* experiments confirm that a reduction in the alkylation of nucleic acids occurs in a cysteine protected tissue, and the authors contend that this reduction can wholly account for the observed protection.

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