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HEPATOTOXICITY OF *N*-METHYLFORMAMIDE IN MICE—I

RELATIONSHIP TO GLUTATHIONE STATUS

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Abstract—In order to investigate the link between hepatotoxicity caused by *N*-methylformamide (NMF) and its ability to deplete hepatic glutathione experiments were conducted in three strains of mouse which differ in their susceptibility towards NMF-induced liver damage. NMF toxicity was measured by changes in plasma levels of sorbitol dehydrogenase and alanine and aspartate transaminases. In BALB/c mice, the most susceptible strain, a hepatotoxic dose of NMF (200 mg/kg) caused a depletion of hepatic glutathione to 21% of control levels 2 hr after drug administration. In CBA/CA and BDF_1 mice the same dose of NMF depleted glutathione to 53% of control levels and did not cause hepatotoxicity. In BALB/c mice depletion of hepatic glutathione by pretreatment with buthionine sulfoximine decreased the hepatotoxic dose threshold of NMF from 150 mg/kg to 100 mg/kg. Conversely, pretreatment of mice with cysteine or *N*-acetylcysteine protected against both glutathione depletion and NMF-induced hepatotoxicity. The results are in accordance with the suggestion that the hepatotoxicity of NMF is associated with its metabolism to an intermediate which reacts with glutathione.

N-Methylformamide possesses antineoplastic activity against certain murine tumours [1, 2] and is a hepatotoxin [3, 4]. Hepatotoxic doses of NMF‡ deplete hepatic glutathione in mice [2]. Recently a major urinary metabolite of NMF has been identified as *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine in rodents and man [5]. The formation of this metabolite involves oxidation of the NMF molecule and reaction with glutathione, presumably in the liver. On the basis of these findings it appears that NMF is metabolised to a reactive intermediate, the proximate or ultimate hepatotoxic species, which reacts with glutathione.

In this paper experiments are described which test the hypothesis that the ability of NMF to cause liver damage and its potential to deplete hepatic glutathione are mechanistically related. To that end the extent of liver damage caused by NMF in three different strains of mouse was compared with the degree of which NMF depleted hepatic glutathione. Furthermore, the influence of treatments which modulate hepatic glutathione levels on the expression of NMF-induced hepatotoxicity was studied. The overall objective of these experiments was to contribute to the understanding of the mechanism of toxicity of this drug and possibly to suggest modalities of treatment which might minimise the toxicities experienced by patients in clinical trials of NMF [6–8].

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‡ Abbreviations used: NMF: *N*-methylformamide; ALT: L-alanine aminotransferase; AST: L-aspartate aminotransferase; SDH: sorbitol dehydrogenase; GSH: glutathione, GSSG: glutathione disulfide.

MATERIALS AND METHODS

Animals and treatments. Male BALB/c, CBA/CA and BDF_1 mice (19–25 g) were purchased from Bantin and Kingman, Hull, U.K., housed on wire mesh and allowed free access to tap water and food (Heygate 13 breeding diet, Pilsbury's Ltd., Birmingham, U.K.). Mice were kept for at least 7 days under an alternating 12 hr light/dark cycle before the experiments. Glutathione, cysteine hydrochloride and *N*-acetyl-L-cysteine were obtained from Sigma Chemical Co., Poole, U.K. NMF was purchased from Aldrich Chemical Co., Gillingham, U.K., and distilled before administration. (±)-Buthionine sulfoximine was prepared by M. D. Threadgill according to Griffith [9]. Agents were administered i.p. after dissolution in isotonic saline (injection volume: 0.2 ml), except *N*-acetylcysteine which was given p.o. Pretreatment schedules for the following three agents were chosen on the basis of published work: buthionine sulfoximine [10]; cysteine [11]; *N*-acetylcysteine [12].

Measurement of hepatic glutathione, urinary thioethers and thioesters and plasma levels of SDH, ALT, AST. Hepatic total glutathione levels (GSH + GSSG) and, in some cases, GSSG were determined spectrophotometrically according to Tietze [13] as described by Akerboom and Sies [14]. Thioethers/thioesters in the urine were measured using Ellman's reagent, after alkaline hydrolysis of ether or ester bonds [15]. The activity of SDH in plasma was assayed according to Rose and Henderson [16]; AST and ALT plasma concentrations were determined as described by Kachamar and Moss [17].

Statistical analysis. Plasma enzyme activities were compared using the Mann–Whitney U-test, hepatic glutathione levels were compared using Student's *t*-test.

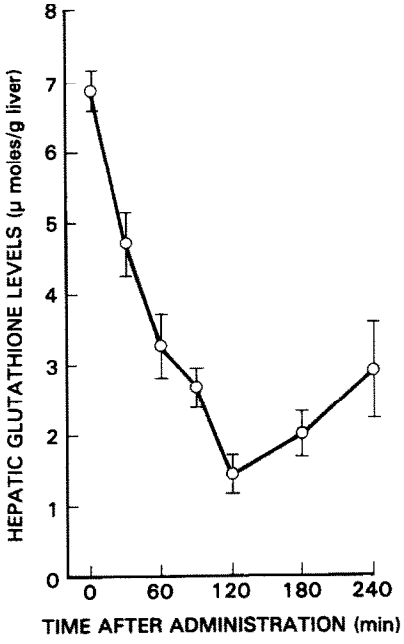

Fig. 1. Time course of depletion of total hepatic glutathione (GSH + GSSG) in BALB/c mice after administration of NMF (200 mg/kg). In some experiments GSSG was determined and levels were never greater than 10% of total glutathione. Values are the mean ± S.E. of 4 mice.

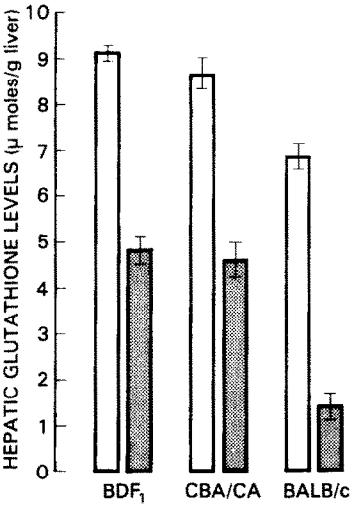

Fig. 2. Strain difference in depletion of hepatic glutathione in mice 2 hr after administration of NMF (200 mg/kg). Open bars, levels observed in mice which received saline; dotted bars, levels in mice which received NMF. Values are the mean ± SE of 4 mice.

RESULTS

NMF caused a profound depletion of hepatic glutathione in the BALB/c mouse (Fig. 1). A single dose of 200 mg/kg, one quarter of the single dose LD_{10} in this strain of mouse [4], caused a decrease of glutathione to a nadir of 21% of control levels 2 hr after drug administration. Hepatic GSSG levels were not raised. Concomitantly urinary excretion of thioethers and/or thioesters was increased from 0.5 ± 0.1 to 1.3 ± 0.1 μmoles *N*-acetylcysteine equivalents/μmol creatinine (mean ±SE, $N = 4$, $P < 0.01$) within 24 hr after NMF administration. Figure 2 shows that there was a difference between strains of mouse in the extent to which NMF decreased hepatic glutathione levels. In both CBA/CA and BDF_1 mice the levels of hepatic glutathione 2 hr after administration of NMF were 53% of control values, significantly less depletion than seen in BALB/c mice ($P < 0.05$). Even though the time course of glutathione depletion was not studied in CBA/CA and BDF_1 mice this result suggests a strain difference in NMF-induced glutathione depletion. There was also a strain difference in susceptibility of mice towards NMF-induced hepatotoxicity as reflected by increased plasma levels of the enzymes SDH, ALT and AST. The dose threshold at which NMF caused hepatotoxicity was between 100 mg/kg and 200 mg/kg in the BALB/c mouse, between 200 mg/kg and 400 mg/kg in the CBA/CA mouse and between 400 mg/kg and 800 mg/kg in the BDF_1 mouse (Table 1).

In order to characterise further the role of glutathione in the genesis of the liver lesion induced by NMF, hepatotoxicity was assessed in BALB/c mice which were pretreated with buthionine sulfoximine 4 hr before administration of NMF. In preliminary experiments it was established that buthionine sulfoximine depleted hepatic glutathione to a nadir of 19% of control values 4 hr after administration. This treatment rendered the normally innocuous dose of 100 mg/kg NMF potently hepatotoxic (Fig. 3). Indeed, mice injected with 100 mg/kg NMF preceded by buthionine sulphoximine were killed after 8 hr because many appeared moribund. By way of contrast, most mice treated with much larger doses of NMF, although showing evidence of liver damage, survived for 24 hr. Conversely, treatment of BALB/c mice with cysteine or *N*-acetylcysteine diminished the glutathione depletion evoked by NMF (Fig. 4) and markedly reduced the hepatotoxicity caused by NMF (Fig. 5). Figure 5 demonstrates the effect of *N*-acetylcysteine and cysteine on the toxicity caused by 200 mg/kg NMF. *N*-Acetylcysteine also protected partially the livers of mice exposed to 400 mg/kg NMF (results not shown).

DISCUSSION

The following three conclusions presented in this paper support the contention that the ability of NMF to deplete hepatic glutathione stores and the mechanism by which it causes hepatotoxicity are linked: (i) both phenomena displayed a similar marked strain difference, (ii) glutathione depletion by pretreatment with buthionine sulfoximine prior to NMF administration exacerbated toxicity, (iii) pretreatment of mice with the thiol compounds cysteine or *N*-acetylcysteine protected their livers against both NMF-induced damage and glutathione depletion.

In an accompanying report we demonstrate that NMF is metabolised *in vivo* and *in vitro* to species which are bound covalently to and are incorporated into hepatic proteins [18]. In *in vitro* incubations the binding to microsomes was totally abolished in the presence of glutathione. These observations and the

Table 1. Plasma activities of SDH in different strains of mouse challenged with NMF (mean ± SE, number of experiments in brackets)

Mouse strain	SDH plasma activities (units/l)* after administration of				
	Saline	NMF 100 mg/kg	NMF 200 mg/kg	NMF 400 mg/kg	NMF 800 mg/kg
BALB/c	42 ± 3 (12)	75 ± 3 (15)	5812 ± 437 (14)	7974 ± 593 (6)	—
CBA/CA	25 ± 3 (6)	38 ± 4 (3)	179 ± 19 (6)	1771 ± 63 (5)	—
BDF_1	28 ± 5 (4)	33 ± 4 (3)	34 ± 12 (6)	32 ± 10 (5)	1349 ± 1008 (6)

* Measured 24 hr after drug administration. Changes in ALT and AST values were very similar to the changes in SDH values shown here.

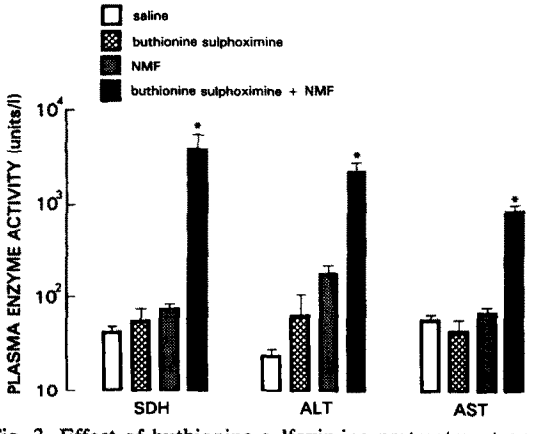

Fig. 3. Effect of buthionine sulfoximine pretreatment on plasma activities of SDH, ALT and AST 8 hr after administration of NMF (100 mg/kg) compared with enzyme activities in BALB/c mice 24 hr after administration of saline, NMF alone or buthionine sulfoximine followed by saline. Buthionine sulfoximine (1600 mg/kg) was administered 4 hr before NMF (or saline). It has been shown previously in BALB/c mice that enzyme activities after hepatotoxic doses of NMF are near control levels for 10 hr after administration, beyond which they rise dramatically [3]. Values are the mean ± SE of 6 to 8 BALB/c mice; * indicates values are significantly different from mice which received saline ($P < 0.001$, Mann-Whitney U-test).

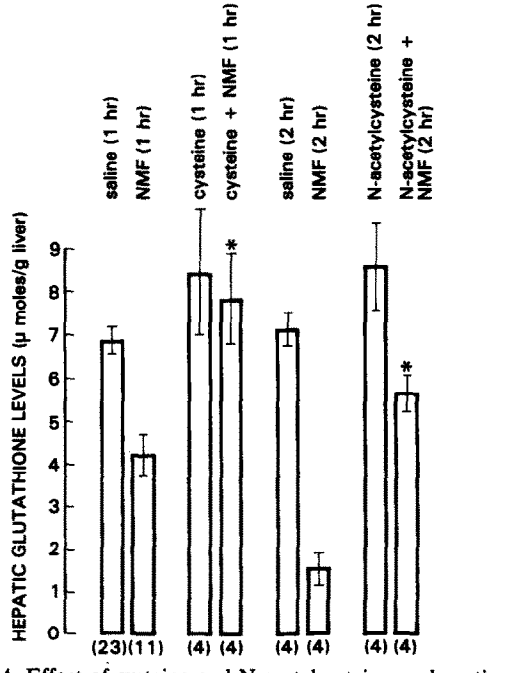

Fig. 4. Effect of cysteine and N-acetylcysteine on hepatic glutathione levels 1 or 2 hr after administration of NMF (200 mg/kg). Cysteine hydrochloride (300 mg/kg) was administered i.p. 10 min before and 20 min after saline or NMF. *N*-Acetylcysteine (1200 mg/kg) was given p.o. 20 min before saline or NMF. Number of observations in brackets; * indicates values (mean ± SE) are significantly different from values obtained in the mice which received NMF alone ($P < 0.01$, Student's *t*-test).

finding that an oxidation product of NMF is excreted as *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine in the urine [5] strongly suggest that NMF is metabolized to a reactive, potentially toxic, intermediate which could be responsible for the hepatic glutathione depletion described here. However, at present the chemical nature of the toxic metabolite of NMF is unknown. One possibility is the formation of methyl isocyanate (Me—N=C=O) as intermediate, which would explain the toxicity observed. An alternative explanation for the protective effect of glutathione is that a metabolite of NMF causes oxidation of protein thiols or lipid peroxidation. However, this is unlikely as hepatic levels of GSSG were not raised as a consequence of NMF administration.

In the phase 1 clinical trials of NMF, hepatotoxicity was one of the complications encountered [6–8]. The results presented here suggest that this toxicity might be obviated by the coadministration of thiol containing compounds. It is possible, though, that such a treatment reduces the antineoplastic efficacy of NMF. This has to be investigated. Also, it remains to be seen whether the general malaise and nausea which turned out to be the major clinical manifestation of toxicity caused by NMF [7, 8, 19] is a consequence of the impairment of liver functions or a central effect.

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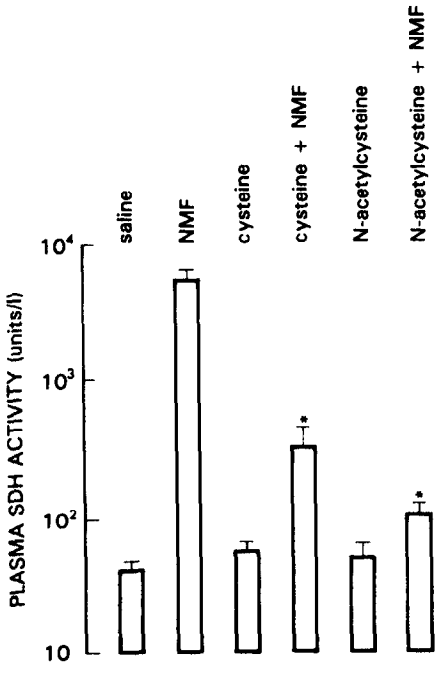

Fig. 5. Effects of cysteine and *N*-acetylcysteine on plasma SDH concentrations 24 hr after administration of saline or NMF (200 mg/kg). Cysteine and *N*-acetylcysteine were given as described in the legend of Fig. 4. Values are the mean ± SE of 6 to 8 BALB/c mice; * indicates values are significantly different from mice which received NMF alone ($P < 0.05$, Mann-Whitney U-test). Measurement of ALT and AST plasma concentrations afforded results virtually identical with those shown here for SDH.

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