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RAPID COMMUNICATION

THE EFFECT OF METHOTREXATE ON HEPATIC LEVELS OF REDUCED GLUTATHIONE IN MICE

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Methotrexate (MTX) is used clinically in the treatment of cancer, psoriasis, and rheumatoid arthritis [1,2]. In humans, two types of liver damage are observed in both high- and low-dose MTX regimens [1-5]. Both the low- and high-dose regimens have been associated with elevated levels of serum glutamic oxaloacetic acid transaminase (SGOT) in up to 60% of patients [1,2]. These asymptomatic elevated SGOT levels are transient and resemble a mild hepatitis. Repeated exposure to low levels of MTX, as treatment for psoriasis or rheumatoid arthritis, imposes a risk of chronic liver fibrosis and ultimately a cirrhosis indistinguishable from alcoholic cirrhosis [1-3].

Despite the clinical findings, MTX has only been shown to be hepatotoxic to rats following lifetime exposure (2 yrs) of massive doses [6]. Short-term exposures (24 wks) have revealed no hepatotoxicity [7]. It is possible, however, that hepatotoxicity previously attributed to MTX alone in humans may be caused by an interaction of a potential hepatotoxin with MTX. Patients taking MTX may self-administer a number of other drugs which are potentially hepatotoxic, such as acetaminophen.

In cultured chick hepatocytes induced for cytochrome P-450 by ß-naphthoflavone, MTX increases the toxicity of acetaminophen [8]. MTX alone decreases the concentration of GSH in these cells, a finding that may contribute to increased acetaminophen toxicity (Lindenthal *et al.*, manuscript in preparation). In the present study we show that MTX alone decreased hepatic reduced glutathione (GSH) in mice. These results may provide insight into MTX-mediated hepatotoxicity in humans.

MATERIALS AND METHODS

Animals. Male C57BL6 mice (21 days old) were purchased from Charles River Laboratories (Wilmington, MA). They were housed in polycarbonate cages (5/cage) and allowed *ad lib.* access to food (RMH 3000 Lab Chow, Agway, Waverly, NY) and water.

Animal treatment. Two different MTX treatment regimens were employed: (i) short-term treatment with high doses of MTX and (ii) long-term treatment with low doses of MTX. Protocol (i) involved the treatment of mice with MTX at 10, 25 and 50 mg/kg (6 animals in each group) by intraperitoneal injection (i.p.) on 2 consecutive days. Mice were killed 24h after the second injection. The control group of mice (5 animals) received i.p. injection of 0.9% saline. Protocol (ii) involved the treatment of mice (12 animals per group) with MTX at 5 or 10 mg/kg i.p. twice a week for 4 weeks. Mice were killed 24h after the final injection. The control group of mice received i.p. injection of 0.9% saline. At sacrifice, blood was collected from each mouse, allowed to clot, and then centrifuged; the serum was stored at -20° until time of analysis. Livers were removed, weighed, and immediately frozen in a methanol/dry ice mixture and then stored at -60° until time of analysis.

Chemicals. Methotrexate was a gift from Lederle Laboratories, Pearl River, NY. GSH and mercaptosuccinic acid were obtained from the Sigma Chemical Co., St. Louis, MO; monochloroacetic

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acid was obtained from the Eastman Kodak Co., Rochester, NY; 1-heptanesulfonic acid (HPLC grade), methanol (HPLC grade) and all other chemicals (ACS grade) were purchased from Fisher Scientific, Fairlawn, NJ.

Reduced glutathione. Mouse hepatic GSH levels were determined (i) by the fluorometric procedure of Hissin and Hilf [9] as described previously [10], and (ii) by HPLC coupled with electrochemical detection as described by Stein et al. [11]. The reverse phase ion pair chromatography was performed isocratically using a 5 μm pore size Microsorb C_{18} (25 cm x 5 mm) column (Rainin Instrument Co., Woburn, MA) with a mobile phase of 0.1 M monochloroacetic acid and 3.3 mM 1-heptanesulfonic acid (pH 3.0), methanol (95:5). GSH was detected by electrochemical oxidation (+0.15 V) with a gold-mercury electrode. Mercaptosuccinic acid was used as an internal standard.

Serum glutamate pyruvate transaminase (SGPT) activity. SGPT activity was determined using a standard kit from Sigma Diagnostics (St. Louis, MO) and expressed as Sigma-Frankel Units/ml, means ± SEM of pooled serum samples (N = 3) made up of serum pooled from three to four mice within the same treatment group.

Statistical analyses. Samples were compared by Student's t-test.

RESULTS AND DISCUSSION

The results of the short-term high-dose MTX treatment on hepatic levels of GSH are shown in Fig. 1. These doses caused no increase in SGPT activity, indicating no hepatotoxicity (results not shown). Significant decreases were noted in mouse hepatic GSH levels following short-term treatment with 25 and 50 mg/kg MTX whether measured fluorometrically (Fig. 1b) or by HPLC (Fig. 1c). However, liver weight was not affected (Fig. 1a). The absolute amount of hepatic GSH decreased by the MTX treatments was the same in both assays (Fig. 1d); however, the fluorometric assay yielded a higher amount of GSH, probably due to the non-specific reaction with certain biogenic amines [12]. The results of the long-term low-dose MTX treatment are shown in Fig. 2. Although there was no toxicity as measured by body weight (Fig. 2a), liver weight (Fig. 2b), or SGPT activity (Control 21 ± 7, 5 mg MTX/kg 17.8 ± 5.1, 10 mg MTX/kg 14.5 ± 2.7), a significant decrease in hepatic GSH levels was observed with 10 mg/kg MTX whether measured fluorometrically (Fig. 2c) or by HPLC (Fig. 2d). The absolute decrease in GSH at the 10 mg/kg dose was very similar by the two methods: 2.36 μmol GSH/g fresh wt (fluorometry) vs 1.90 μmol GSH/g fresh wt (HPLC).

In summary, we have shown that both acute and chronic treatment of male C57BL6 mice with MTX resulted in a significant decrease in hepatic GSH levels without any other overt signs of toxicity. GSH plays a crucial role in protecting the cell against hepatotoxins, by conjugating with reactive electrophiles and reacting with peroxides generated during the development of hepatic damage [13]. Leszczynska-Bisswanger and Pfaff [14,15] have shown, in isolated hepatocytes, that phorone-mediated decreases in GSH are associated with increased uptake of MTX into those cells. It is possible, therefore, that MTX by lowering GSH may accelerate its own uptake, thereby compounding the overall effect on hepatic GSH levels. Also, Berge et al. [16] have reported a decrease in the hepatic GSH content due to MTX administration in rats fed a choline-deficient diet. MTX, by decreasing hepatic GSH levels, may lower the threshold dose of potential hepatotoxins. These results may help to explain the two types of hepatotoxicity associated clinically with MTX therapy.

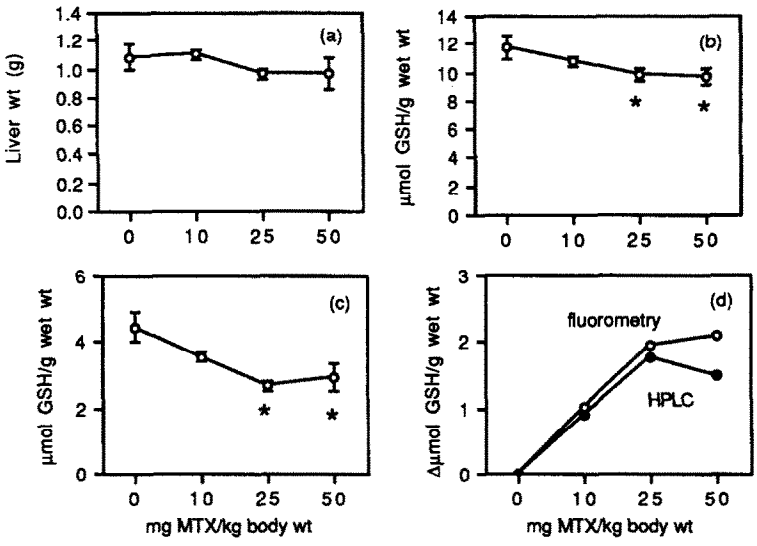

Fig. 1. Effect of short-term MTX treatment of mice on hepatic weight and GSH. Male C57BL6 mice were injected daily i.p. with MTX for 2 consecutive days. Mice were killed 24h after the final dose. Livers were removed and weighed (a). Hepatic GSH was determined by fluorometry (b) and HPLC (c) as described in Materials and Methods, with a comparison of the two methods shown in (d). Values are means ± SEM, N = 5 for control, N = 6 for MTX groups. *Significantly different from control, $P \leq 0.05$.

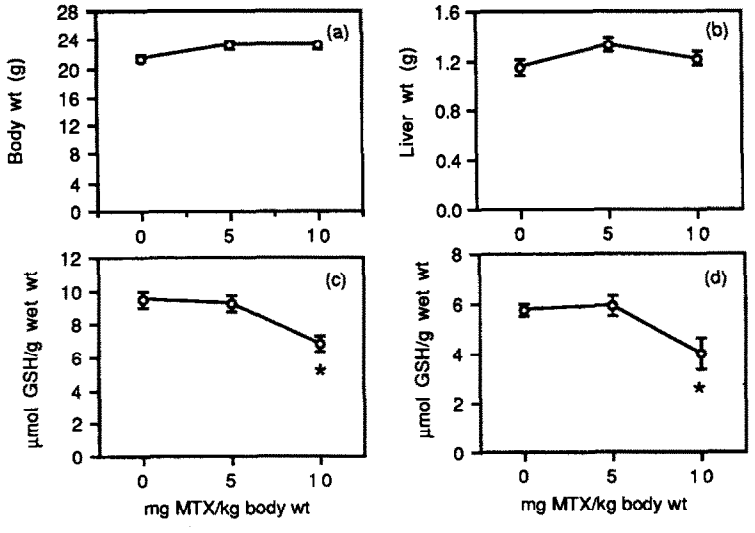

Fig. 2. Effect of long-term MTX treatment of mice on body weight, hepatic weight and GSH. Male C57BL6 mice were injected i.p. with MTX at 5 or 10 mg/kg body wt, twice a week for 4 weeks. Mice were killed 24h after the final dose, body weight was measured (a), and livers were removed and weighed (b). Hepatic GSH was determined by fluorometry (c) and HPLC (d) as described in Materials and Methods. Values are means ± SEM, N = 12 in: control and 5 mg/kg, N = 10: 10 mg/kg. *Significantly different from control, $P < 0.001$ (c) and $P < 0.01$ (d).

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