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Phenylalanine-induced leucopenia in genetic and dichloroacetic acid generated deficiency of glutathione transferase Zeta

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ABSTRACT

Glutathione transferase Zeta (GSTZ1-1) is identical to maleylacetoacetate isomerase and catalyses a significant step in the catabolism of phenylalanine and tyrosine. Exposure of GSTZ1-1 deficient mice to high dietary phenylalanine causes a rapid loss of circulating white blood cells (WBCs). The loss was significant ($P < 0.05$) after 2 days and total WBCs were reduced by 60% after 6 days. The rapid loss of WBCs was attributed to the accumulation of the catabolic intermediates maleylacetoacetate or maleylacetone (MA) in the circulation. Serum from GSTZ1-1 deficient mice treated with phenylalanine was cytotoxic to splenocytes from normal BALB/c mice and direct incubation of normal splenocytes with MA caused a rapid loss of viability. Dichloroacetic acid (DCA) has been used therapeutically to treat lactic acidosis and is potentially of use in cancer chemotherapy. Since DCA can inactivate GSTZ1-1 there is a possibility that long-term treatment of patients with DCA could cause GSTZ1-1 deficiency and susceptibility to oxidative stress and phenylalanine/tyrosine-induced WBC loss. However, although we found that DCA at 200 mg/(kg day) causes a severe loss of hepatic GSTZ1-1 activity in BALB/c mice, it did not induce WBC cytotoxicity when combined with high dietary phenylalanine.

1. Introduction

Glutathione transferase Zeta (GSTZ1-1) is identical to maleylacetoacetate isomerase the penultimate step in the catabolism of phenylalanine and tyrosine [1,2]. Mice that are genetically deficient in GSTZ1-1 suffer from significant oxidative stress and are very sensitive to acetaminophen toxicity as a result of low hepatic reduced glutathione (GSH) concentrations [3–5]. In addition, GSTZ1-1 deficient mice experience significant liver damage and a striking loss of blood leukocytes when phenylalanine is included in their diet [4]. GSTZ1-1 deficiency can be chemically induced by exposure to dichloroacetic acid (DCA) [6,7]. Previous studies have shown that DCA is dechlorinated and converted to glyoxylate by GSTZ1-1 in the presence of GSH [8,9]. DCA can also act as a suicide substrate for GSTZ1-1 where the S-chloromethylglutathione intermediate reacts with the thiol of Cys16 to form an inactive substrate–enzyme complex [10,11]. In humans the extent of GSTZ1-1 inactivation by DCA is correlated with different GSTZ1-1 genotypes and the most common isoform is among the most sensitive to inactivation [10]. In mice and rats, exposure to 50–200 mg DCA/(kg day) causes a severe GSTZ1-1 deficiency [6,7,12]. DCA is a contaminant of chlorinated drinking water [13,14] and has been regarded as a possible human carcinogen as it can cause tumours in rats and mice [15–17]. Despite the concerns about its potential carcinogenicity DCA has been used in humans as an experimental drug to alleviate lactic acidosis [18]. DCA inhibits pyruvate dehydrogenase kinase (PDK) that in turn regulates pyruvate dehydrogenase and the metabolism of lactate through the citric acid cycle. Some patients receiving long-term DCA therapy have experienced peripheral neuropathy that may result from demyelination [18,19]. Recently DCA has been proposed as a potential low toxicity cancer therapy [20]. Most solid tumours manifest the "Warburg effect" that restricts metabolism through the citric acid cycle and maintains ATP production by anerobic glycolysis. DCA can reactivate oxidative phosphorylation and slow proliferation of cancer cell lines. In addition, DCA can induce apoptosis in cancer cells and lower the metastasis of A429 human lung tumours in nude rats [20]. It is thought that the reactivation of mitochondria and apoptotic pathways is mediated by the inhibition of PDK but other possibilities need to be further explored.

Because of the potentially increased use of DCA in the treatment of lactic acidosis and cancer there is a possibility that patients may become GSTZ1-1 deficient and susceptible to the loss of leukocytes that accompanies phenylalanine or tyrosine exposure in GSTZ1 knockout mice [4]. A generalized loss of leukocytes would be an undesirable side effect for any therapy. We have now investigated the loss of leukocytes in GSTZ1 knockout mice in more detail and

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compared their response to mice made deficient in GSTZ1-1 by exposure to DCA.

2. Materials and methods

2.1. Reagents

All chemicals were obtained from Sigma unless otherwise noted. Maleylacetone (MA) was prepared by a previously described method [21]. Fumarylacetone (FA) was obtained from the Chemistry Centre (Perth, Australia). Chlorofluroacetate was gift from Dr. M.W. Anders, Department of Pharmacology and Physiology, University of Rochester.

2.2. Mice

The *GstZ1* deficient mice were developed in the BALB/c strain and have been previously characterized [4]. The wild type BALB/c and *GstZ1* knockout mice were housed under a 12-h light/dark cycle and fed standard mouse chow. Phenylalanine (3% solution) was administered to mice in drinking water. DCA was diluted in saline, neutralized with NaOH and was injected IP daily (200 mg/kg) for 5 days prior to phenylalanine treatment and daily thereafter. Wild type male BALB/c mice used in the DCA/phenylalanine experiment were 22–24 weeks old.

2.3. Splenocyte viability assays

Splenocyte viability was assessed by incubating splenocytes from male BALB/c mice with serum obtained from 3% phenylalanine treated male BALB/c or *GSTZ1*$^{-/-}$ mice or by incubating with MA, succinylacetone (SA) or HGA. Target splenocytes were depleted of erythrocytes with PharmLyse™ buffer (BD Biosciences, NJ, USA). Assays were performed in U-bottomed 96-well tissue culture plates with splenocytes at a density of 5×10^6 cells/mL in complete RPMI supplemented with 10% fetal calf serum. Serum from mice treated with phenylalanine for at least 6 days was obtained by cardiac puncture and allowed to clot at room temperature for 30 min and centrifuged at $2000 \times g$ for 10 min. Serum was added to cells to a final concentration of 50% (v/v). Cells were incubated at 37 °C/5%CO_2. For the serum cytotoxicity assays, splenocyte suspensions were stained with propidium iodide and Annexin-V-FITC (BD Biosciences, NJ, USA). For cytotoxicity assays with MA, SA and HGA, cells were stained with 7-amino-actinomycin D. Cells were analysed on a BD LSR flow cytometer (BD Biosciences, NJ, USA).

2.4. Hematological analysis

A 20-μL sample of blood was collected by tail vein puncture and diluted 10-fold in ACD buffer (100 mM trisodium citrate, 70 mM citric acid, 100 mM glucose, pH 4.5). The diluted samples were analyzed on an ADVIA 2120 hematology analyzer (Siemens, Victoria, Australia) that had been specifically calibrated for BALB/c blood cells.

2.5. Blood glutathione determination

Total glutathione was measured by a previously described method [22]. Although we measured total blood glutathione, this primarily represents GSH derived from erythrocytes. The blood glutathione levels are expressed in terms of hemoglobin rather than protein. Blood was collected by retro-orbital bleeding, and 100 μL samples were lysed with 1 mL H_2O. A 100-μL sample of lysed blood was mixed with 5 mL Drabkin's solution and kept on ice until hemoglobin estimation. The remaining 1 mL of blood lysate was combined with 1 mL 10% sulfosalicylic acid and allowed to stand for 15 min on ice, followed by centrifugation at $14{,}000 \times g$ for 10 min at 4 °C. The acid supernatant was frozen on dry ice and stored at −70 °C until assayed.

2.6. Enzyme assays

GSTZ1-1 activity was determined as the rate of transformation of chlorofluroacetic acid (CFA) to glyoxylate [8,9]. CFA was used as a substrate as unlike DCA it does not inactivate GSTZ1-1 [10]. Western blots were undertaken with anti-GSTZ1-1 serum generated in previous studies [4].

3. Results

3.1. Time course of leukocyte loss

Although a previous study noted a profound loss of nucleated blood cells from the circulation of *GstZ1* knockout mice fed 3% phenylalanine in their drinking water, the time course of this response was not determined [4]. We undertook differential blood cell counts at 48 h intervals on wild type and *GstZ1* knockout mice that were fed either water or water containing 3% phenylalanine. The GSTZ1-1 deficient mice fed 3% phenylalanine progressively lost white blood cells (WBCs) over a period of 6 days (Fig. 1A). The loss of WBC was significant after 2 days ($P < 0.05$). Wild type BALB/c mice were unaffected by phenylalanine and *GstZ1* knockout mice receiving water had a normal blood cell profile. The total WBC count in Fig. 1A is shown for simplicity and similar decreases were seen in other WBC subsets (Table 1). This response is similar to the previous result obtained by Lim et al. (2004) [4] after several weeks of phenylalanine exposure. In contrast to the dramatic loss of leukocytes, red blood cell (RBC) numbers were unaffected (Fig. 1B).

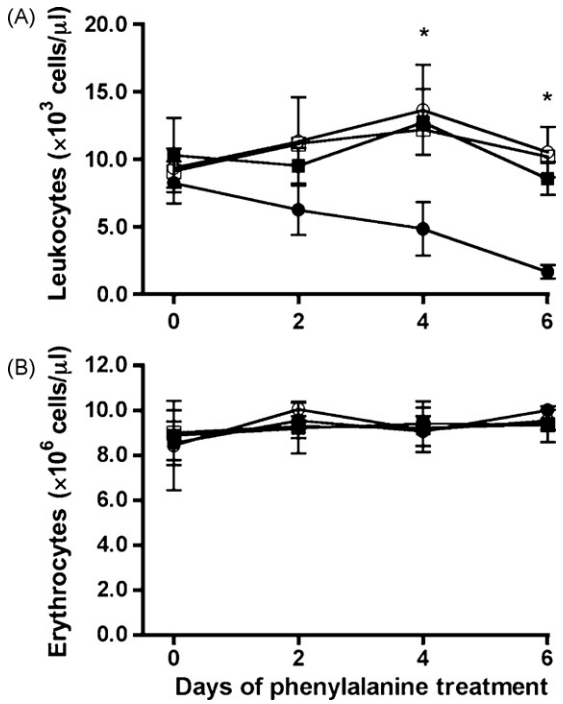

Fig. 1. White blood cell (A) and red blood cell (B) counts from mice fed with water or water containing 3% phenylalanine. The mice were 16–22-week females, BALB/c wild type (□), wild type plus Phe (■), GstZ1$^{-/-}$ (○), GstZ1$^{-/-}$ plus Phe (●). Data shown represent mean values ± S.D. with $n = 5$ per group. *$P < 0.005$ wild type plus Phe vs GstZ1$^{-/-}$ plus Phe.

Table 1
Differential blood cell counts from wild type and GstZ1$^{-/-}$ mice treated with 3% phenylalanine in their drinking water for 6 days.

	WT + Phe	*GstZ1*$^{-/-}$ + Phe
Total erythrocytes[a]	8.92 ± 0.84	9.57 ± 0.49
Total leukocytes[b]	6.05 ± 1.02	2.42 ± 0.84*
Lymphocytes[b]	2.16 ± 0.34	1.04 ± 0.48*
Neutrophils[b]	3.45 ± 0.79	1.16 ± 0.54*
Monocytes[b]	0.22 ± 010	0.07 ± 0.05*
Eosinophils[b]	0.16 ± 0.06	0.13 ± 0.10
Basophils[b]	≤0.02 ± 0.005	≤0.02 ± 0.007

Values are means ± S.D.
[a] 10^6 cells/μL
[b] 10^3 cells/μL.
* $P < 0.0005$, unpaired Student's *t*-test $n = 8$ mice per group.

3.2. Cytotoxicity of serum from GSTZ1-1 deficient mice fed phenylalanine

To determine if the loss of WBC resulted from a defect in the WBC from *GstZ1* knockout mice or from the buildup of a toxic intermediate in the circulation we incubated splenocytes obtained from wild type BALB/c mice with serum obtained from either wild type mice or *GstZ1* knock out mice that had been fed 3% phenylalanine in their drinking water for at least 6 days. The data in Fig. 2 show that serum from phenylalanine treated *GstZ1*$^{-/-}$ mice caused a significant increase in the proportion of dead propidium iodide positive and Annexin V negative splenocytes compared to serum from wild type BALB/c mice. These data indicate that when GSTZ1-1 deficient mice are treated with phenylalanine they accumulate a compound in their blood that is toxic to nucleated blood cells. Furthermore, this toxicity is not restricted to cells that have a deficiency of GSTZ1-1.

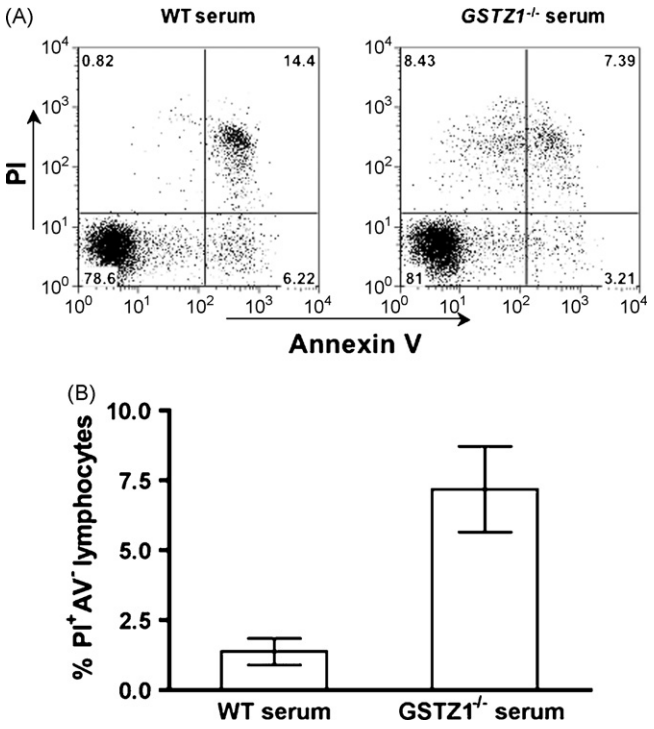

Fig. 2. The cytotoxicity of serum from mice fed with phenylalanine. (A) A representative flow cytometry plot of erythrocyte depleted splenocytes incubated for 24 h with serum from wild type or GstZ1$^{-/-}$ mice fed with 3% Phe in drinking water. Splenocytes were stained with Annexin V and propidium iodide. (B) Percent non-apoptotic non-viable splenocytes (Pi^+ Annexin V^-), mean ± S.D. with $n = 9$ per group. $P < 0.00001$ in a paired *t*-test.

3.3. The cytotoxicity of phenylalanine catabolic intermediates

Fig. 3 shows the phenylalanine/tyrosine catabolic pathway and the intermediates that might accumulate if a deficiency of GSTZ1-1 blocks the isomerization of maleylacetoacetate (MAA) to fumarylacetoacetate. The accumulation of MAA would be expected but it is unstable and can be decarboxylated to maleylacetone or reduced and decarboxylated to SA. Because of its instability MAA cannot be readily synthesized *in vitro* and is not readily available. The cytotoxicity of homogentisate, MA and SA was evaluated with splenocytes isolated from wild type BALB/c mice (Fig. 4). After incubation with different intermediates splenocyte viability was estimated by 7-AAD staining and flow cytometric analysis. Homogentisate and SA did not prove to be particularly cytotoxic even at concentrations as high as 500 μM. In contrast 50 μM MA produced significant cytotoxicity after 48 h ($P < 0.0001$). At a concentration of 500 μM, only 4.9 ± 1% of cells remained viable after 12 h.

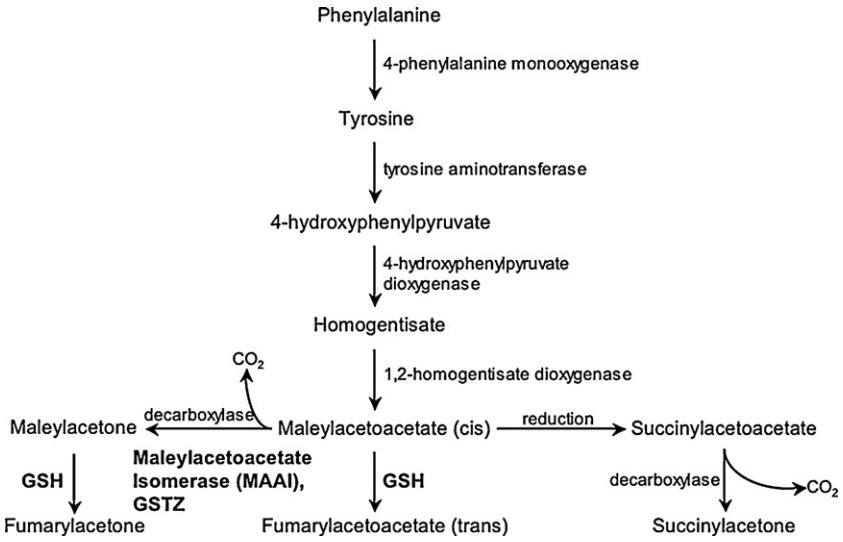

Fig. 3. The enzymatic steps and intermediates in the phenylalanine and tyrosine catabolic pathway preceding the GSTZ1-1 catalysed isomerization of maleylacetoacetate to fumarylacetoacetate.

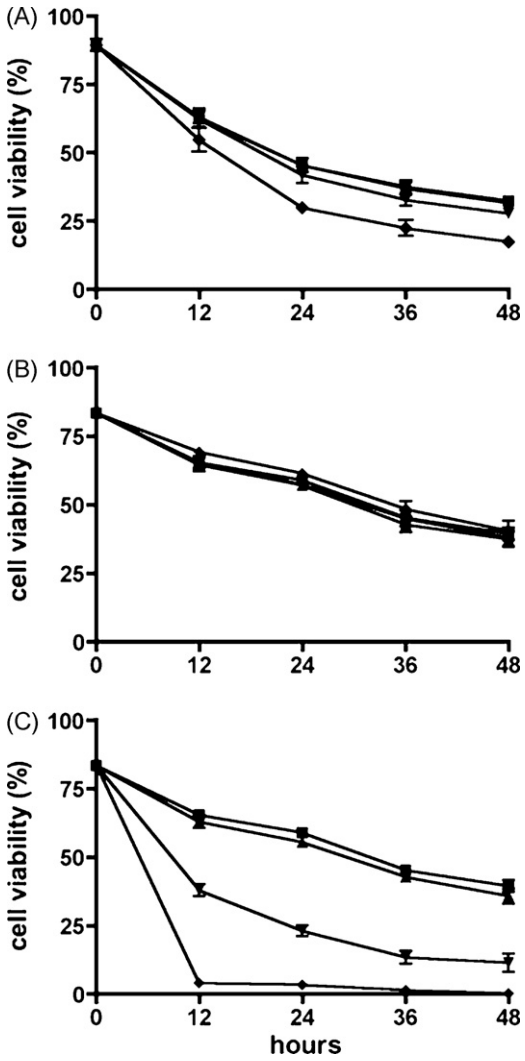

Fig. 4. The cytotoxicity of tyrosine catabolites. Splenocytes were obtained from male BALB/c mice (8–10 weeks of age) and incubated with 0 μM (■), 5 μM (▲), 50 μM (▼), and 500 μM (◆) (A) homogentisate, (B) succinylacetone and (C) maleylacetone. Viability was assessed by flow cytometry and 7-AAD staining. The data are the mean ± S.D. from 5 wild type BALB/c mice.

3.4. Oxidative stress resulting from phenylalanine catabolism

In a previous study we found that the liver of *GstZ1* knockout mice was subjected to constitutive oxidative stress [5]. To determine if this oxidative stress extends to the blood and if it is elevated by ingestion of phenylalanine we determined glutathione concentrations in whole blood. Because total blood glutathione is predominantly GSH derived from RBCs the results are expressed as μmol/g hemoglobin. The data in Fig. 5 show that even with a normal diet GSTZ1-1 deficient mice tend to have a lower blood glutathione concentration than wild type mice. The inclusion of 3% phenylalanine in their drinking water for 7 days caused a significant decrease ($P < 0.0001$) in total glutathione in the blood of *GstZ1* knockout mice. This result indicates that the oxidative stress observed in the liver is also evident in the blood and may contribute to the loss of WBC after phenylalanine ingestion.

3.5. DCA induced GSTZ1-1 deficiency

Our previous studies have shown that GSTZ1-1 can be irreversibly inactivated by DCA [10,11,23]. Consequently it is possible that treatment of lactic acidosis or cancer with DCA may

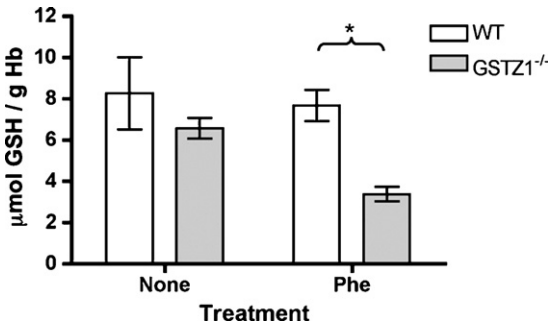

Fig. 5. Total blood glutathione (GSH + GSSG). Wild type (open bars) and GstZ1$^{-/-}$ mice (shaded bars) were provided with plain water or water containing 3% phenylalanine. Data are the mean ± S.D. with $n = 5$ per group. *$P < 0.0001$.

cause a severe deficiency of GSTZ1-1. Such a deficiency may predispose an individual to oxidative stress and loss of WBCs if they consumed a diet that was high in phenylalanine or tyrosine. In order to examine this possibility, we treated wild type BALB/c mice with DCA (200 mg/(kg day)) for 5 days and then with DCA and phenylalanine for 6 more days at which point blood was collected for hematological analysis and liver samples were assayed for GSTZ1-1 activity. After 5 days this DCA treatment almost completely eliminated GSTZ1-1 activity measured with CFA as a substrate (Fig. 6). The DCA treatment also substantially lowered the amount of GSTZ1-1 protein detectable by Western blot (Fig. 6). We then treated similar mice with 3% phenylalanine in their drinking water for a further 6 days. The data in Fig. 6 and Table 2 indicate that although DCA essentially abolished hepatic GSTZ1-1 activity, the addition of phenylalanine to the diet did not cause the severe loss of WBCs that is observed in *GstZ1* knockout mice. In addition we did not find a decrease in blood or liver glutathione in the DCA/Phe treated mice (Fig. 7) indicating that they are not under the same level of oxidizing stress as occurs in genetically determined GSTZ1-1 deficiency.

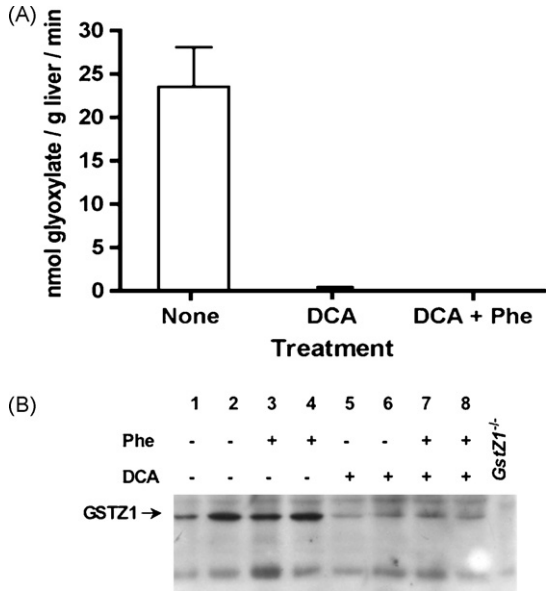

Fig. 6. The inactivation of hepatic GSTZ1-1 in wild type BALB/c mice treated with DCA. Panel A, the dehalogenase activity of GSTZ1-1. The data represent the mean and standard deviation of measurements from 5 mice in each group. Panel B, a Western blot of liver cytosol from mice treated with DCA and phenylalanine. Liver cytosol from a GstZ$^{-/-}$ mouse is included as a control. The non-specific proteins above and below the Zeta band provide a loading control.

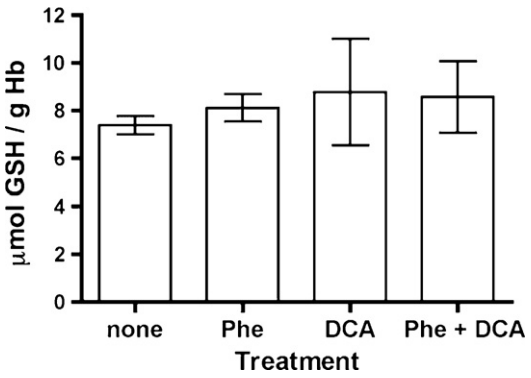

Fig. 7. Total blood glutathione (GSH + GSSG). Wild type BALB/c mice were treated with phenylalanine and or DCA as described in the text. The data are the mean ± S.D. of between 4 and 6 mice in each group.

Table 2
Differential blood cell counts from wild type BALB/c mice pre-treated with DCA (200 mg/(kg day)) for 5 days and with DCA and 3% phenylalanine in their drinking water for another 6 days.

	DCA	DCA + Phe
Total erythrocytes[a]	9.62 ± 0.73	10.73 ± 0.76
Total leukocytes[b]	8.52 ± 1.19	7.95 ± 1.03
Lymphocytes[b]	3.24 ± 0.61	3.64 ± 0.91
Neutrophils[b]	4.48 ± 1.28	3.61 ± 0.54
Monocytes[b]	0.53 ± 0.42	0.38 ± 0.18
Eosinophils[b]	0.21 ± 0.07	0.27 ± 0.11
Basophils[b]	≤0.02 ± 0.005	≤0.02 ± 0.005

Values are means ± S.D.
n = 6 mice per group.
[a] 10^6 cells/µL.
[b] 10^3 cells/µL.

4. Discussion

The present data confirm that the exposure of GSTZ1-1 deficient mice to high levels of dietary phenylalanine causes a depletion of circulating WBC and further shows that this depletion is underway within 48 h. GSTZ1-1 is expressed strongly in the liver and hepatocytes are considered to be a major site of phenylalanine and tyrosine catabolism [4]. As GSTZ1-1 is only sparingly expressed in WBC, it was of interest to determine if this response was due to a defect in the WBCs of *GstZ1*$^{-/-}$ mice or due to the accumulation of a toxic intermediate that passes from the liver into the circulation. Our results show that normal splenocytes obtained from wild type BALB/c mice are killed by serum from *GstZ1*$^{-/-}$ mice treated with phenylalanine, indicating that the loss of leukocytes is due to a toxic compound in the circulation and not a defect in the WBC from *GstZ1*$^{-/-}$ mice. It is possible that the splenocytes are killed by a process that is independent of apoptosis as the elevated population of dead cells as determined by propidium iodide staining (PI^+) did not stain for Annexin V. However, further detailed studies are required to resolve this point.

Although it is likely that maleylacetoacetate is the main intermediate that accumulates in the liver of *GstZ1*$^{-/-}$ mice exposed to high levels of dietary phenylalanine it was not possible to evaluate its toxicity directly because it is unstable and is not readily synthesized *in vitro*. As an alternative we tested its decarboxylation product maleylacetone as well its reduction product SA, and homogentisate the immediate precursor of MAA in the phenylalanine catabolic pathway. SA and homogentisate showed only minor levels of toxicity and can therefore be excluded as the cause of leukocyte loss in *GstZ1*$^{-/-}$ mice. High levels of SA are a diagnostic feature of tyrosinemia caused by fumarylacetoactetate hydrolase (FAAH) [24]. Although leukocyte cytotoxicity is not a significant feature of FAAH deficiency, it is of interest that SA has been shown to be a potent immunosuppressive agent [25]. In the present study MA was found to be cytotoxic towards normal mouse splenocytes confirming the previous observation of its cytotoxicity towards a mouse hepatocyte cell line [7]. These results suggest that the dramatic loss of leukocytes from *GstZ1*$^{-/-}$ mice treated with phenylalanine results from the accumulation of MAA or MA. Rats with DCA induced GSTZ1-1 deficiency excrete MA in their urine [6,7] which suggests that MAA accumulating in the liver is released into the circulation and is filtered in the kidney. The point at which MAA is decarboxylated to MA is not clear from the presently available data. MAA and MA are electrophiles capable of alkylating protein thiols [7] and this may be the basis of their cytotoxic effects. The severe depletion of blood glutathione in *GstZ1*$^{-/-}$ mice on normal mouse chow and those supplemented with additional phenylalanine is consistent with this mechanism.

Previous studies have shown that GSTZ1-1 deficiency occurs in rats treated with DCA at concentrations that are used in humans for the treatment of lactic acidosis [6,7]. As MA and some MAA was found in the urine of these rats we considered the possibility that DCA induced GSTZ1-1 deficiency may confer susceptibility to the phenylalanine mediated toxicity and the loss of leukocytes that we have observed in *GstZ1*$^{-/-}$ mice. This could be a significant adverse side effect in humans receiving DCA therapy for lactic acidosis or cancer. In the present study in mice, DCA treatment caused the almost complete inactivation of GSTZ1-1. However, in contrast to *GstZ1*$^{-/-}$ mice fed additional phenylalanine, the DCA/phenylalanine treated mice showed no loss of WBC. This result suggests that the very low residual GSTZ1-1 was sufficient to avoid the accumulation of MA to cytotoxic levels. In a previous study [4] we reported high levels of SA in the serum of *GstZ1*$^{-/-}$ mice treated with phenylalanine. It s likely that this apparent SA also includes MA as the assay we used is indirect and detects components in serum that inhibit δ-aminolevulinate dehydratase [26]. In the present study, mice treated with DCA and phenylalanine were normal and had no detectable SA/MA in their serum. Circulating MA may also be buffered by the alkylation of erythrocyte GSH. We noted that erythrocyte GSH was significantly depleted in *GstZ1*$^{-/-}$ mice treated with phenylalanine and MA may not reach cytotoxic levels if high levels of erythrocyte GSH remain as occurs in DCA treated mice.

The present data suggest that subjects receiving long-term DCA therapy are probably not at as great a risk of WBC depletion as might be expected in individuals with a complete genetic deficiency of GSTZ1-1. This conclusion is substantiated by the available data from human clinical trials [18]. So far no human cases of GSTZ1-1/MAAI deficiency have been fully characterized. Although DCA treated mice were not susceptible to phenylalanine-induced toxicity, there may be species differences that suggest caution in ongoing human trials of DCA therapy. Since *GstZ1*$^{-/-}$ mice present signs of oxidative stress when on a normal diet [5], it is possible that patients receiving long-term DCA therapy may also suffer from oxidative stress that would be increased when consuming a diet rich in phenylalanine and or tyrosine. In addition, individuals with glucose 6 phosphate dehydrogenase (G6PD) deficiency can suffer from hemolytic anemia when exposed to oxidative stress and could potentially be at risk if treated with DCA. G6PD deficiency occurs relatively frequently in some ethnic groups [27].

Acknowledgments

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