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The toxic milk mouse does have elevated hepatic metallothionein mRNA

The mouse mutant termed toxic milk (*tx*) has the interesting phenotype of hepatic copper accumulation which is associated with production of copper-deficient milk by the mutant dam. The deficiency of copper in the milk can be severe enough to cause death in the suckling pups, although this is not always the case [1]. The excess hepatic copper has been reported to be associated with metallothioneins (MTs) [2]. In previous work, we determined the concentrations of metallothionein-I mRNA in the livers of *tx*/*tx*, *tx*/+ and +/+ animals, during development and after administration of copper or zinc. Our conclusions were that the levels of MT mRNA and response to induction were normal in mutant pups before the accumulation of copper in the liver, which commences in the second postnatal week [3]. In adult *tx*/*tx* animals we examined only two males and two females, and found in these animals that the concentration of MT-I mRNA was approx. 10-fold higher in the mutants. The difference was so striking that we considered this to be a sufficiently large sample. We concluded that the increase in MT-I mRNA was a secondary consequence of the accumulation of copper.

In contrast with this, recent work by Koropatnick and Cherian [4] failed to find an elevation of MT-I or MT-II mRNA in the *tx*/*tx* mouse liver, despite a marked accumulation of MT. They concluded that their results could be explained by a difference in stability of MT in the mutant, and provided some evidence of increased stability of the protein. This result was suggested as evidence that the primary defect involved 'altered mRNA translation and/or altered proteolysis of MT (either by heritable changes in MT structure or proteolytic activity in liver)' [4].

In view of the important difference this result makes to the interpretation of the nature of the primary defect, we re-examined our evidence for elevated MT-I mRNA in the *tx*/*tx* liver. Our original data [3] were based on dot-blot analysis of mRNA; here we present Northern-blot analysis of RNA from another group of animals, i.e. normal and *tx*/*tx* adult male mice at 60 and 150 days of age (two animals in each group). The blots provide a more graphic illustration of the differences that we observe. To confirm the genotype of the animals, we determined hepatic copper concentrations in a portion of the same liver used for RNA isolation. The mean hepatic copper concentrations were determined to be 16 and 588 μg/g dry wt. for the normal and *tx*/*tx* animals respectively at 60 days, and 14 and 815 μg/g for +/+ and *tx*/*tx* respectively at 150 days, which is similar to previous data [3,4]. RNA was isolated from the same livers, electrophoresed on a formaldehyde/agarose gel, blotted and probed with ^{32}P-labelled MT-I cDNA probe as described previously [5]. After only a 2 h exposure the MT-I mRNA in the four *tx*/*tx* mice gave an intense signal (Figure 1a, lanes 7, 8, 11 and 12), whereas MT-I mRNA was undetectable in the normals (DL strain) (lanes 5, 6, 9, 10). The level of MT-I mRNA in the mutants is similar to that found in copper-induced animals (lanes 3, 4). The loading of RNA was similar for all samples, as shown by removing the MT-I signal with boiling 0.1% SDS and re-probing using the house-keeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPD) (Figure 1b; [6]); note some residual signal from MT-I in the lower half of Figure 1(b). Taken together with our previous results, we have data that show that in eight *tx*/*tx* animals the mRNA is at least 10-fold higher than in the equivalent number of normals, and, in addition, in other experiments we have always found elevated mRNA in adult toxic milk mice (at least six other animals; results not shown). Since the level of hepatic MT mRNA is not elevated in the liver of newborns before copper accumulation [3], it is reasonable to conclude that the copper accumulation is responsible for the increase in MT mRNA, although a detailed study of the correlation of copper concentration with MT mRNA during development has not been carried out. Thus we conclude that the very high level of metallothionein present in the liver of the *tx*/*tx* animals is perfectly in accord with the amount of MT mRNA in these animals; there is no need to postulate a defect of MT

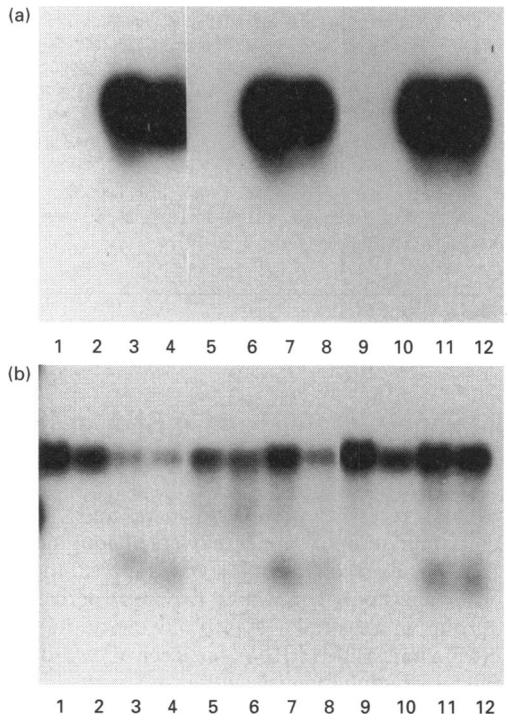

Figure 1 Northern-blot analysis of hepatic RNA from normal and *tx*/*tx* mice

Total liver RNA was isolated from mice and electrophoresed on 1.5%-agarose gels, blotted on to Hybond N+ membrane and probed with an MT-I cDNA or GAPD probe as described previously [5,8]. (**a**) MT-I mRNA: lanes 1, 2, BALB/c, 60 days; lanes 3, 4, BALB/c, 60 days, injected intraperitoneally twice at 24 h intervals with 2 mg/kg Cu as copper acetate; lanes 5, 6, +/+, DL strain, 60 days; lanes 7, 8 *tx*/*tx*, 60 days; lanes 9, 10, +/+, DL strain, 150 days; lanes 11, 12, *tx*/*tx*, 150 days. (**b**) The same blot stripped and re-probed with GAPD; note residual MT-1 mRNA signals in lower half.

degradation. The fact that differences in half-life were found [4] can possibly be explained by the high copper content of the MTs in the mutant liver influencing the stability of the protein. Our findings are supported by the work of Yamada et al. [7] with the LEC rat, a mutant with many of the properties of the *tx* mouse, and the copper accumulation is found to be associated with both elevated MT and MT mRNA.

The striking difference in the reported MT-I mRNA levels is surprising. We note that Koropatnick and Cherian [4] used a genomic MT probe which includes both MT-I and MT-II genes. The exons of these genes together comprise only about 6% of the total probe (600 out of 9.8 kb), so this will give a very weak signal compared with the cDNA probe that we used, and might lead to other artefactual signals if the genomic DNA contains repeat elements.

We conclude therefore that there is no primary abnormality of metallothionein stability in the *tx/tx* mouse, and the primary defect most likely involves a defect of copper excretion similar to that found in Wilson's disease, and MT is induced by the accumulating copper.

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1 Rauch, H. (1983) J. Hered. **74**, 141–144
2 Rauch, H., Dupuy, D., Stockert, R. J. and Sternlieb, I. (1986) in Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine (Rotilio, G., ed.), pp. 304–306, Elsevier, Amsterdam
3 Mercer, J. F. B., Grimes, A. and Rauch, H. (1992) J. Nutr. **122**, 1254–1259
4 Koropatnick, J. and Cherian, M. G. (1993) Biochem. J. **296**, 442–449
5 Mercer, J. F. and Wake, S. A. (1985) Nucleic Acids Res. **13**, 7929–7943
6 Piechaczyk, M., Blanchard, J. M., Marty, L., Dani, C., Panabieres, F., El Sabouty, S., Fort, P. and Jeanteur, P. (1984) Nucleic Acids Res. **12**, 6951–6963
7 Yamada, T., Suzuki, Y., Ague, T. and Matsumoto, K. (1992) Biochim. Biophys. Acta **1131**, 188–191
8 Mercer, J. F. B., Livingston, J., Hall, B. K., Paynter, J. A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemenack, D. and Glover, T. W. (1993) Nature Genet. **3**, 20–25

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Metallothionein protein and mRNA in the *toxic milk* mouse

An autosomal recessive mutation (*toxic milk*, or *tx*) with increased accumulation of copper in livers of adult mice was first identified by Dr. Harold Rauch [1], who supplied these mutants and the wild-type stock from which they were derived to us and another group in Australia. A 10-fold elevation in metallothionein (MT) and MT-1 mRNA has been shown by Mercer et al. [2] in livers of adult *tx* mice. We have reported high hepatic Cu-MT, with little change in MT-1 plus MT-2 mRNA in *tx* mouse liver [3]. Mercer et al. [4] suggest that the copper accumulation is closely associated with both elevated MT and MT mRNA. Since the *tx* mutant mice used in both these studies are from the same source, we suggest that apparent differences may be due to environmental factors, including diet.

Our animals are maintained on a diet enriched in zinc (114 p.p.m.) and designed to enhance conditioning. As a consequence, basal MT mRNA may be elevated in certain tissues. Differences in dietary metals could contribute to the discrepancy in relative MT mRNA levels reported by us and Mercer et al. [4].

Zinc is essential for basic cellular functions [5] and is anti-inflammatory in some immune cells [6]. Stress caused by a variety of factors induces MT in rodent liver [7,8]. High dietary zinc can decrease the intestinal absorption and hepatic accumulation of copper. As a consequence, dietary zinc may also alleviate or delay stress-related changes caused by copper: in aged *tx* mice (7–9 months old) we observe an increase in MT mRNA that was not apparent in younger animals ([3]; and results not shown). Since a number of agents (including metals, hormones, cytokines, and factors released during cell injury) can induce MT synthesis in liver, conditions affecting production of all these factors should be considered. This is especially important for *tx* mice, because copper is not as good an inducer of MT synthesis as zinc in mammalian systems, and its deficiency in pregnant mice does not affect fetal liver MT expression in rats [9].

We believe that it is not entirely clear that enhanced MT gene transcription can completely account for elevated MT protein. We have reported a trend toward slightly increased MT mRNA at 60 days of age in homozygous *tx* mice; however, variability between animals did not allow us to attribute any significance to this difference [10]. This trend did not account for a 35-fold increase in hepatic MT protein and an 8–10-fold increase in hepatic copper [10].

The mouse genomic probe complementary to both mouse MT-1 and MT-2 [3] gives the same qualitative results as a cDNA probe specific for mouse MT-1 (results not shown). As suggested by Mercer et al. [4], the signal is weaker. However, it does not appear that specificity was affected by the repeat elements present in the probe.

We agree with Mercer and co-workers that increased hepatic MT stability in *tx* mice does not imply that this is the only cause of the *tx* defect. It is conceivable (as discussed by us [3] and by Mercer et al. [4]) that high copper content increased the stability of the protein. However, previous reports suggest that Cd-MT is approx. 4-fold more stable than Cu,Zn-MT (e.g. [11]). In our hands, hepatic *tx* MT induced by and associated with cadmium, and MT bound primarily to copper, both exhibit enhanced stability [3].

The primary abnormality in the *tx* mouse is probably excess accumulation of copper in the liver, leading to increased MT. Indeed, we have observed that there is diminished MT protein between birth and weaning in *tx* mice, compared with high developmental hepatic expression in wild-type animals. MT mRNA at that early stage of development is also low, in good correlation with MT protein ([10]; J. Koropatnick, G. Stephenson, M. G. Cherian and J. Pearson-Sharpe, unpublished work). This suggests that low copper is associated with low MT mRNA and protein levels in early development in *tx* mice. A defect in copper transport similar to that reported in Wilson disease, perhaps to the fetus, would provide a logical explanation. However, it could also be true that the *tx* defect involves both abnormal copper transport and altered MT metabolism (perhaps an effect of abnormally high intracellular copper on cellular processes). Both would contribute to enhanced intracellular accumulation of copper and metallothionein.

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1 Rauch, H. (1983) J. Hered. **74**, 141–144
2 Mercer, J. F., Grimes, A. and Rauch, H. (1992) J. Nutr. **122**, 12–19
3 Koropatnick, J. and Cherian, M. G. (1993). Biochem. J. **296**, 443–449
4 Mercer, J. F. B., Paynter, J. A. and Grimes, A. (1994) Biochem. J. **304**, 317–318
5 Vallee, B. L. and Falchuk, K. H. (1993) Physiol. Rev. **73**, 79–105
6 Leibbrandt, M. E. I. and Koropatnick, J. (1993) Toxicol. Appl. Pharmacol. **124**, 72–81