Increased Metallothionein in Mouse Liver, Kidneys, and Duodenum during Lactation

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Lactation-induced increases in cadmium absorption and retention have been demonstrated in mid-lactating mice, but no systematic measurements of endogenous metal-binding protein concentrations during lactation have been reported. Using Cd/hemoglobin radioassay, this study detected significant increases in metallothionein (MT) concentrations in liver (4-fold), kidneys (2-fold), and duodenum (2-fold), but not jejunum, of mouse dams on days 13 and 20 of lactation. These increases occurred in the absence of cadmium exposure and were specific to the lactation period; dams 5 days after weaning showed MT levels that were similar to those of nonpregnant (NP) mice. Similarly, Northern blot analyses of livers from lactating mice demonstrated that MT mRNA concentrations in maternal liver during mid-lactation were 6-fold higher than those observed 5 days after pups were weaned. Gel filtration of final supernatants from the Cd/hemoglobin assay confirmed that the Cd-binding molecule induced during lactation was indeed metallothionein. In addition, chromatographic analyses of cytosols from tissues taken from dams administered small amounts of Cd (66 ng/mouse) showed that the trace amounts of Cd absorbed through the maternal gastrointestinal tract during mid-lactation were also bound to the MT. These results indicate MT induction in mouse dams occurs as a physiological consequence of lactation, requiring no external stimulus. This induced MT participates in binding low levels of dietary cadmium consumed by the dam. During lactation, elevated maternal MT may affect pathways for essential trace metals as well as sequester toxic metals harmful to the neonate. Multiparous humans may have increased risk of accumulating environmental Cd.

Key Words: metallothionein; cadmium exposure; environmental cadmium; lactation; liver; kidneys; duodenum; mouse.

A number of classic studies have shown that the intestinal uptake of various essential metal ions increases in pregnant and/or lactating animals, reflecting increased demand for these elements by the developing fetus and neonate. For example, absorption of oral copper, zinc (Lucis et al., 1972; Williams et al., 1977), and iron (van Dijk et al., 1983) increases by 50–200% during late gestation and lactation. In addition to the 3 transition metal ions, the absorption of calcium has been shown to markedly increase in pregnant and lactating rats (Halloran and DeLuca, 1980; Toverud et al., 1976). As with essential metals, toxic environmental metal ions appear to increase in their intestinal absorption in pregnant and/or lactating animals. For example, lactating rats retain 3 times as much orally administered lead as do nulliparous animals (Keller and Doherty, 1980). The absorption of cadmium in pregnant and lactating animals increases by 2- to 3-fold (Bhattacharyya, 1983; Bhattacharyya et al., 1982; Pietrzak-Flis et al., 1978; Whelton et al., 1993, 1997), reaching a peak at mid-lactation (Bhattacharyya et al., 1981). The study reported here was designed to determine whether there were lactation-induced increases in the metal-binding protein metallothionein (MT) that might play a role in the changes in intestinal absorption and subsequent distribution patterns for dietary cadmium in the maternal animal.

Cadmium is a widely distributed environmental pollutant (Friberg et al., 1986). Environmental exposure levels of cadmium are relatively low, with daily intake ranging from 4 to 60 μg in humans (Friberg et al., 1974, 1986; Tahvonen, 1996). However, the cumulative retention of cadmium, due to its long biological half-life in mammalian systems (Bhattacharyya et al., 1986; Friberg et al., 1986; Goyer, 1991), makes this metal one that demands close scrutiny as a potential environmental health concern. Cadmium exposure has been implicated as one cause for the outbreak of Itai-Itai disease in Japan (Nogawa et al., 1990). This disease is characterized by osteomalacia and renal tubular dysfunction, and it affects mainly postmenopausal women with a history of multiple child-bearing. The particular susceptibility of this population to Itai-Itai disease may be partially attributed to increased maternal absorption of dietary cadmium during pregnancy and lactation (Wang et al., 1994; Webster, 1988). In addition to the renal and bone responses, cadmium has more recently been linked to carcinogenic and teratogenic changes in tissues (Copras and Antonio, 1998; Mahalik et al., 1995; Piasek and Laskey, 1999; Waalkes and Oberdörster, 1990; Webb et al., 1988). On the basis of...
these and other studies, cadmium is considered a human carcino

The mechanisms for increased intestinal absorption and re
tention of both essential and toxic metal ions during pregnancy
and lactation are still under study, but metal-binding proteins,
including MT, clearly influence metal absorption pathways
(Chan et al., 1993; Chmielnicka and Sowa, 1996; Suzuki et al.,
1990; Webb, 1986). Because MT plays an important role in the
binding and metabolism of zinc and copper, as well as cad-
mium, in all developmental stages (Borghesi and Lynes, 1996;
Cherian and Chan, 1993; Clarkson, 1995; Kägi, 1993; Kern et al.,
1981; Liu et al., 1996), and has been shown to be elevated in
pregnancy (Chan and Cherian, 1993; Chan et al., 1993;
Chmielnicka and Sowa, 1996; Payan et al., 1990; Suzuki et al.,
1990), we have examined whether the concentrations of this
protein show change in maternal tissues during lactation. Anal-
yses for MT include gel filtration chromatography and hemo-
globin-binding assay techniques, utilizing the protein’s low
molecular weight (~6000 Da), characteristic elution from
Sephadex G-50/-75 gel filtration columns (V0, [elution vol-
ume]/V0, [void volume] ~ 1.8–2.3), and relative heat stability
(Eaton and Cherian, 1991; Suzuki, 1992). Possible regulation
of cellular MT at the MT mRNA level is explored through
Northern blotting techniques.

In the current study, changes were examined in maternal MT
levels in tissues from lactating mouse dams in the absence of
any external stimulus. In addition, we investigated the binding of
trace amounts of 109Cd to MT in mouse tissues after oral
administration of carrier-free 109Cd to evaluate the role of MT
in Cd pathways during lactation. Concentrations of MT mRNA
were determined in livers from mid-lactating and post-lactating
mouse dams with no cadmium exposure. The results demon-
strate that increases in MT protein and mRNA concentrations
occur in mouse dams as a physiological response to lactation.
In addition, this MT is an important part of the mechanism of
handling low levels of oral cadmium by the lactating dam.

MATERIALS AND METHODS

Preparation of tissues for determination of MT concentrations during lactation. B6CF1/Anl mice (Argonne National Laboratory, Argonne, IL) were maintained on Wayne Lab Blox diet (Wayne Feeds, Continental Grain, Libertyville, IL) and tap water (acidified to pH 2.3 with HCl to inhibit the growth of Pseudomonas species). Protocols for animal use were approved by the Argonne National Laboratory Animal Care and Use Committee. Animals were maintained in the Argonne animal facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Mice were 78 days old and weighed 18–22 g at the start of the study. They were mated by housing 2 females with 1 male. After a 6-day mating period, females were separated from the males and housed individually in plastic cages. Starting on the 18th day after the initiation of mating, mice were checked daily for one week for delivery of pups. Day 1 of lactation (L 1) is the day following delivery. At days 2 to 3 of lactation (L 2 and L 3), litters were adjusted to 7 pups per dam. Pups were weaned on L 20.

Lactating dams were assigned to 1 of 3 groups of 10 animals each. These groups were designated L 13, L 20, and W 5, indicating the dams were to be sacrificed on L 13, L 20, or day 5 after weaning (W 5), respectively. To relate the results of this study to our previous one, which demonstrated increased absorption of cadmium on specific days of lactation (Bhattacharyya et al., 1981), one subgroup of mice was treated according to the same fasting and gavage procedures used in our earlier study. Thus, half of the mice from each group were fasted overnight (18 h), gavaged with 0.2 ml of double-deionized water, and sacrificed 3 days later, as described previously (Bhattacharyya et al., 1981). The other 5 animals in each group served as nongavage controls and were not fasted or gavaged. This protocol was carried out to investigate the possibility that the gavage procedure and the stress of overnight fasting, particularly in lactating dams, might itself have induced MT, as suggested by Bremner and Davies (1975) and Oh and co-workers (1978).

Two additional groups of mice were the nonpregnant (NP) controls. A zero-time NP group was sacrificed on the same day that mating of animals in the lactating groups was initiated. To track possible changes in MT concentra-
tions with age in the young adults used in this study, a second NP control group was sacrificed 37 days later. (With the 6-day mating period employed, delivery dates spanned a week’s time, and dams with early delivery dates were assigned to the L-20 group. Consequently, the 37-day NP mice were interme-
diate in age (~2 days) between the L-13 and L-20 animals.) Each NP control group was also subdivided into gavage and nongavage subgroups, as above.

Mice were sacrificed by intraperitoneal injection with pentobarbital (12 mg/mouse, ip, Nembutal, Abbott Laboratories, Abbott Park, IL). Liver, kid-
eys, duodenum, and jejunum (defined as the first 5 cm and the next 10 cm of the small intestine, respectively) were removed. The lumens of the small intestinal segments were thoroughly rinsed with 10 mM Tris HCl, pH 7.4, in 0.9% NaCl. The duodenum and jejunum were immediately placed on an ice-cold stainless steel plate. Each segment was cut longitudinally, spread open, and the mucosal layer scraped off by using the sharp edge of a micro-
scope slide, leaving behind only the transparent muscle layer. All tissue samples were immediately frozen inside a cryogenic tube by immersion in liquid nitrogen and stored at ~20°C. These intestinal tissues were later homogen-
enated for determination of MT by Cd/hemoglobin assay, as described below. Tissue homogenates and supernatants were prepared in cold, 10 mM Tris HCl buffer containing 5 mM 2-mercaptoethanol (2-ME) (TMEM buffer), at pH 7.4 or 8.6, as specified in the particular procedure.

Determination of metallothionein by Cd/hemoglobin assay. The MT content of clear supernatants (10,000 × g, 20 min, 4°C) from tissue homog-
enates was analyzed by a modified version of the Cd/hemoglobin assay (Eaton and Cherian, 1991). Because of concern for the stability of MT (Minkel et al., 1980), 2-ME was added as an SH-protector. The 2-ME did not appreciably increase the assay background (data not shown). To assure accuracy of the Cd/hemoglobin assay, preliminary assays were carried out with each tissue homogenate to determine the amount of tissue that gave MT values in the linear portion of the tissue dilution curve. Samples were stored overnight at 4°C in buffer with 5 mM 2-ME prior to the final assay. This method of storage was found necessary in order to retain the full amount of MT in the samples. To confirm the Cd-binding protein as MT, the final Cd/hemoglobin assay supernatant was chromatographed, as below.

The 109Cd content of the final supernatant was used to calculate the MT concentration in the tissue, according to Eaton and Cherian (1991). A well-type gamma-ray spectrometer (Beckman Gamma 310 or Beckman Gamma 8000) was used to measure 109Cd. The counting efficiency of the 88 KeV gamma radiation of 109Cd was 74–78% as determined with a 109Cd reference source (New England Nuclear, Boston, MA). Radiation safety protocols were super-
vised by the Environmental Safety and Health Division of Argonne National Laboratory.

Preparation of cytosol fractions after oral 109Cd administration during lactation. L-10 and nonpregnant B6CF1/Anl mice analogous to those de-
scribed above were fed by gavage with 66 ng of cadmium as 109CdCl2 (86 μCi per mouse; carrier-free 109Cd, 1.3 Ci/mg; New England Nuclear, Boston, MA) in 0.2 ml water after an overnight fast. Three days later (L 13 for lactating dams), the mice were sacrificed and samples of duodenum and liver, the major early deposition sites for orally administered cadmium, were taken as de-

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in age (B6CF1/Anl mice (129 days old, 26–28 g) with a series of subcutaneous columns (Sigma Chemicals). Analyzed as above. Molecular weight markers were used to calibrate the supernatant fractions obtained from Cd/hemoglobin assays, as well as in the chromatographic elution pattern of 109 Cd in tissues from the lactating mice was also separated on Sephadex. Both elution profiles had a peak with V_e/V_o consistent with its being bound to MT.

Gel filtration column chromatography. Cd-binding molecules present in the supernatant fractions obtained from Cd/hemoglobin assays, as well as in the 109Cd-containing cytosol was chromatographed on a Sephadex G-75 column. The remainder was heat-treated once (60°C, 2 min), centrifuged (40,000 × g for 20 min) to remove heat-labile precipitates, and re-chromatographed on Sephadex G-75, to determine whether gentle heating might change the chromatographic profile. Gel filtration column chromatography. Cd-binding molecules present in the supernatant fractions obtained from Cd/hemoglobin assays, as well as in the 109Cd-containing cytosol was chromatographed on a Sephadex G-75 column. The remainder was heat-treated once (60°C, 2 min), centrifuged (40,000 × g for 20 min) to remove heat-labile precipitates, and re-chromatographed on Sephadex G-75, to determine whether gentle heating might change the chromatographic profile.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Liver</th>
<th>Kidney</th>
<th>Duodenum</th>
<th>Jejunum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Time</td>
<td>Nongavage</td>
<td>11.5 ± 0.8</td>
<td>5.6 ± 0.5</td>
<td>11.9 ± 1.1</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>11.4 ± 1.1</td>
<td>6.6 ± 0.6</td>
<td>11.2 ± 1.8</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>Nongavage</td>
<td>14.5 ± 1.1</td>
<td>10.9 ± 0.1</td>
<td>20.9 ± 1.2</td>
<td>9.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>15.9 ± 1.7</td>
<td>11.7 ± 0.8</td>
<td>17.3 ± 0.9</td>
<td>10.4 ± 0.4</td>
</tr>
<tr>
<td>Lactation day 13</td>
<td>Nongavage</td>
<td>62.2 ± 1.3</td>
<td>24.1 ± 1.1</td>
<td>45.0 ± 4.8</td>
<td>14.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>66.2 ± 5.3</td>
<td>20.5 ± 1.3</td>
<td>47.1 ± 2.7</td>
<td>13.1 ± 1.2</td>
</tr>
<tr>
<td>Lactation day 20</td>
<td>Nongavage</td>
<td>40.0 ± 2.3</td>
<td>21.7 ± 1.7</td>
<td>38.7 ± 3.0</td>
<td>11.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>37.4 ± 2.9</td>
<td>21.6 ± 0.5</td>
<td>36.8 ± 1.2</td>
<td>10.5 ± 0.7</td>
</tr>
<tr>
<td>5 days postweaning</td>
<td>Nongavage</td>
<td>8.6 ± 0.7</td>
<td>11.7 ± 0.6</td>
<td>18.8 ± 2.4</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>13.7 ± 0.7</td>
<td>10.2 ± 0.6</td>
<td>19.9 ± 1.3</td>
<td>9.9 ± 1.0</td>
</tr>
</tbody>
</table>

*Values are mean (± SE) and n = 5 animals per group, except as indicated. MT in duodenum and jejunum was determined in mucosa only. Values significantly different (p < 0.05, ANOVA + LSD test) from nonpregnant group, which comprised mice 37 days older than 0-time mice and similar in age (± 2 days) to mice at lactation days 13 and 20. *n = 4.

Values significantly different (p < 0.05, ANOVA + LSD test) from 0-time group (nonpregnant mice sacrificed at the first day of mating).

**RESULTS**

**Effect of Fasting/Gavage Protocol Alone on Tissue MT Concentrations**

This component of our MT evaluation included samples prepared from mice that had undergone the same fasting/gavage protocol used in the previous baseline study (Bhattacharyya et al., 1981) as well as those that had not. As shown in Table 1, there were no significant differences between MT concentrations in fasted/gavaged vs. control animals within any group of mice.
**MT Concentration in 0-Time and NP Controls**

Tissues of nonpregnant animals contained basal levels of MT ranging from about 6 to 12 μg MT/g wet-weight tissue (zero-time samples; Table 1). Mean concentrations of MT in tissues of the NP controls, which were 37 days older than the zero-time animals, appeared to be 50–100% higher than those of their younger counterparts. However, apparent increases with animal age were consistently statistically significant (p < 0.05) only for the kidney samples.

**MT in Tissues of Lactating and Non-Pregnant Mice as Determined by Cd/Hemoglobin Assay**

As shown in Table 1, significant increases in MT protein concentration occurred in the tissues of mouse dams during lactation. In L-13 animals, the increases were statistically significant (p < 0.05) in liver (4.3-fold), kidneys (2.0-fold), and duodenum (2.4-fold) above those of the NP controls of similar age. By L 20, the MT concentrations of these tissues decreased slightly, but still represented 2-fold increases (p < 0.05) over those of the NP mice. MT increases in the small intestine were specific to the duodenum; concentrations in the jejunum samples from L-13 and L-20 dams were not significantly greater than those of the age-appropriate NP controls. Finally, the increases of MT concentration in affected tissues were specific to the lactation period; by 5 days after weaning, MT concentrations in tissues of the dams returned to levels similar to those of the NP controls.

**Gel Filtration of Final Supernatants from Cd/hemoglobin Assay**

Gel filtration chromatography of standard MT obtained from the liver cytosol of male mice injected with non-radioactive CdCl₂ (to induce MT protein) resulted in the recovery of cadmium in a low-molecular-weight peak at Vₑ/Vₒ = 2.2 (Fig. 1A). The gel filtration profiles of the final supernatants from the Cd/hemoglobin assays for specific groups are also presented. Final supernatants from NP liver samples (Fig. 1D) showed that the main peak of ¹⁰⁹Cd chromatographed in the same position as standard mouse MT (Vₑ/Vₒ = 2.2). For the L-13 (Fig. 1E) and L-20 (Fig. 1F) liver samples, the predominant ¹⁰⁹Cd peak was higher in radioactivity than for the NP sample and again co-chromatographed with standard MT, with minor peaks appearing at Vₑ/Vₒ values of 1.0 (void volume) and 1.65.

For final supernatants obtained from the Cd/hemoglobin assay of L-20 kidney (Fig. 1B) and duodenum (Fig. 1C) samples, similar results were obtained. As in liver samples, the main ¹⁰⁹Cd peak co-chromatographed with standard MT, while minor peaks were observed at Vₑ/Vₒ values of 1.0, 1.3, and 1.65. A pooled and concentrated sample of fractions from the Vₑ/Vₒ = 1.3 peak of the above duodenum sample was incubated with the reducing agent, 2-ME. Gel filtration of this sample resulted in the elution of the entire ¹⁰⁹Cd radioactivity at Vₑ/Vₒ = 2.2 (data not shown).

**Northern Blot Analyses of RNA from Mid-Lactating and Post-Lactating Mouse Liver**

Standard RNA samples extracted from the livers of Cd-treated mice showed a prominent RNA band that hybridized with the MT DNA probe in the Northern-blot analyses (Fig. 2, lane 1). Corresponding bands of MT mRNA were identified in Northern blots (Fig. 2) of RNA samples extracted from the

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**FIG. 1.** Elution of final supernatants from Cd/hemoglobin assays of induced mouse MT (A), of L-20 kidney (B), L-20 duodenum (C), NP liver (D), L-13 liver (E) and L-20 liver (F) samples from Sephadex G75 columns. Kidney and duodenum samples were pooled from 3 mice, necessitated by low ¹⁰⁹Cd radioactivity in samples. Total ¹⁰⁹Cd radioactivity loaded on each column and total recovery (in parenthesis) is: (B) 2679 cpm (96%), (C) 8382 cpm (117%), (D) 4223 cpm (89%); (E) 3185 cpm (87%); and (F) 1499 cpm (75%). Arrow markers indicate elution positions of standards: (a) blue dextran (MW > 2 × 10⁶ Da), (b) chicken ovalbumin (45 kDa), (c) bovine erythrocyte carbonic anhydrase (29 kDa), (d) cytochrome c (12.4 kDa), and (e) aprotinin (6.5 kDa).
livers of individual L-13 (lanes 2–4) and W-5 (lanes 5–7) mouse dams. Densitometric measurements of the autoradiograms showed that the mean liver MT mRNA abundance in L-13 dams (percentage intensity of $^{1}C_{d}$ standard: $58 \pm 13\%$, mean $\pm$ SE, $n = 3$) was 6-fold higher ($p < 0.01$) than in dams 5 days after weaning the pups ($9.6 \pm 4\%$).

MT Complexation of Cadmium in Liver and Duodenum after Intestinal Absorption of Cd

Figure 3 shows representative chromatographic profiles of liver and duodenum cytosolic fractions obtained from lactating mouse dams and corresponding NP controls that were gavaged with trace amounts of $^{109}Cd$ on L 10 and sacrificed on L 13. (Approximately 60% of the whole-organ $^{109}Cd$ was recovered in cytosolic fractions; the fraction containing the nuclei and cell debris contained 20–30% of the $^{109}Cd$.)

In the livers of NP control mice (Fig. 3A), levels of cytosolic $^{109}Cd$ 3 days after GI absorption, were low, but 83% of the measurable cadmium was associated with a protein that co-chromatographed with MT ($V_e/V_o = 2.1$). Heating this liver cytosol at 60°C for 2 min prior to chromatography still afforded 87% recovery of the cytosolic $^{109}Cd$ associated with a peak at $V_e/V_o = 2.2$ (Fig. 3B). The liver cytosol of the NP mouse also appeared to contain minor metal-binding protein(s) of higher molecular weight that were associated with 16–17% of the total recovered $^{109}Cd$ (Figs. 3A and 3B).

For mouse dams on L-13, the amount of $^{109}Cd$ deposited in the liver after oral $^{109}Cd$ increased significantly (Compare ordinate scales of Figs. 3A vs. 3C). Chromatographic profiles showed that $^{109}Cd$ in the L-13 liver cytosol was almost exclusively associated with a peak at $V_e/V_o = 1.7$ (Fig. 3C). Cytosol from the L-13 duodenum (Fig. 3E) contained 91% of the recovered $^{109}Cd$ in the region typical of MT ($V_e/V_o = 2.3$). When these L13 cytosols from liver and duodenum were heated to 60°C as above, 84 and 83% of the cytosolic $^{109}Cd$, respectively, remained soluble in the supernatant. Chromatography of these heat-stable supernatants showed that all of the soluble $^{109}Cd$ in L-13 liver gave an elution profile typical of standard MT ($V_e/V_o = 2.1$; Fig. 3D), and 94% of the L-13 duodenum $^{109}Cd$ retained the same MT peak at $V_e/V_o = 2.3$ (Fig. 3F).
DISCUSSION

The investigation reported here was designed to provide insight into the previously observed 2- to 3-fold increase in the intestinal absorption of low levels of cadmium observed in mouse dams during lactation, and the significant increases in the fraction of intestinally-absorbed cadmium deposited and retained in the kidneys of these dams (Bhattacharyya et al., 1981, 1982; Pietrzak-Flis et al., 1978; Whelton et al., 1993). On the basis of the latter metabolic data, the hypothesis was developed that MT concentrations in mouse dams might increase, at least in the liver, kidneys, and intestines, during lactation. It was reasoned that increased MT concentrations might increase long-term retention of Cd in maternal organs, and increase the release of Cd-MT to blood from the dam’s liver or small intestines, resulting in enhanced deposition of Cd in the kidney.

The results of the present study demonstrate that concentrations of MT, the major cadmium-binding protein in higher organisms, did indeed increase significantly in the liver, kidneys, and duodenum of mouse dams during mid-lactation (Table 1). Because the dams in this study were not administered any agent, such as cadmium, the stimuli for the observed rises in tissue metallothionein levels remain to be explored. The progesterone-induced increases in MT synthesis and Cd toxicity observed in rat-liver cells in vitro by Shimada and coworkers (1997) provide one example of MT expression being under hormonal control.

The increases in MT concentrations reported in this study were specifically associated with the lactation period. Once dams entered the post-lactation period (W 5), MT concentrations returned to levels comparable to those found in NP controls (Table 1) and the MT mRNA levels decreased (Fig. 2). Although the lactation-induced increase in liver MT concentration shown here was accompanied by a 2-fold increase in the weight of the dam’s liver (Bhattacharyya et al., 1981), the striking decrease in MT after weaning (Table 1) occurred in the absence of any decrease in liver weight (Bhattacharyya et al., 1986). The latter study (1986), which used low cadmium concentrations similar to those in this study, further demonstrates that the increased amount of cadmium taken up and retained by the liver of the dam during pregnancy and lactation is not released from that tissue during a 4-week period after weaning, in spite of the precipitous post-weaning declines in MT protein and MT mRNA concentrations demonstrated by the data reported here.

In addition to being lactation-induced, the increase in MT levels also was tissue-specific. Liver and kidney, the main organs that concentrate cadmium in mammals (Bhattacharyya et al., 1981, 1986; Chan and Cherian, 1993; Webb, 1986), exhibited large lactation-dependent MT increases (Table 1). Duodenum, but not jejunum, also showed an increase in MT levels during lactation. The correspondence of the latter MT changes with our earlier finding of increased cadmium retention in duodenum, but not jejunum, of lactating dams after low-level, oral cadmium administration (Bhattacharyya et al., 1981) indicate that the changes in MT protein reported here do correlate with changes in the uptake and retention of dietary cadmium by the dam’s intestine during lactation. Although previous reports indicated stressful conditions can cause MT increases in rats (Bremner and Davies, 1975; Oh et al., 1978), our data (Table 1) show the fasting and gavage treatment of both NP mice and lactating dams in the gavage subgroups did not induce additional MT synthesis.

Furthermore, our results demonstrate the binding and cytotoxicity observed in rat-liver cells in vitro by Shimada and coworkers (1997) provide one example of MT expression being under hormonal control.

The results of a number of studies, including ours, indicate that the presence of MT in a given tissue does not cause that tissue to initially take up more cadmium from the circulation. That is, endogenous MT does not appear to attract cadmium to a given tissue. For example, in the study of Liu and Klaassen (1996), the significantly elevated concentrations of MT in the tissues of MT-I transgenic mice altered neither the extent of intestinal absorption nor the organ distribution of orally administered cadmium compared to wild-type mice. Furthermore, Liu et al. (1996) demonstrated that the initial pattern of cadmium distribution in the various tissues of mice after parenteral administration was the same, independent of whether the mice were wild-type or were totally deficient in MT1 and MT2. In the latter study, however, cadmium was released from tissues and excreted from the bodies of the MT-deficient mice more rapidly than from the wild-type. Similarly, previous experiments have shown that, after a single dose of cadmium administered by gavage during mid-lactation, the percentage of the administered dose present in the liver at 72 h was not significantly greater in the dam than in nonpregnant controls (Bhattacharyya et al., 1981). Only when cadmium was administered continuously in drinking water over 42 days—a duration potentially long enough to reflect increases in tissue retention half-time—were cadmium amounts and concentrations in the liver greater in dams than in the nonpregnant mice.
(Bhattacharyya et al., 1982). These results and those of others (Liu and Klaassen, 1996; Liu et al., 1996) support the hypotheses that (1) endogenous MT can increase the biological halftime of cadmium retention in tissues, and (2) the initial rate of uptake of cadmium by the tissues appears to be independent of endogenous concentrations of MT.

Because the Cd/hemoglobin assay method can detect Cd-binding molecules other than MT (Waalkes and Perantoni, 1986), some of which might increase during lactation, the final supernatants from these assays were further analyzed by gel filtration (Figs. 1B–1F). Results confirmed that from 70–100% of the $^{109}$Cd measured in the Cd/hemoglobin assays did chromatograph in the same position as standard MT (Fig. 1). Furthermore, our measurements of MT mRNA in liver (Fig. 2), showing corresponding changes in MT mRNA concentrations in mid-lactating and post lactating mice, provide additional support to our interpretation that the Cd/hemoglobin analyses did measure true changes in MT protein in the lactating dam (Table 1, Figs. 1–3).

Aggregation of the cysteine-rich MT polypeptide may have been the cause of at least some of the observed Cd bound to higher-molecular-weight proteins in the gel filtration analyses. For example, virtually all of the Cd in the elution profile of the liver cytosol from L-13 dams migrated at a position corresponding to an MT dimer ($V_e/V_o \sim 1.7$; co-chromatographing with molecular weight standard = 12.4 kD) (Fig. 3C). Because the major difference between the L-13 liver cytosol sample and the liver supernatant from the Cd/hemoglobin assay ($V_e/V_o = 2.2$) was the heating step used in the latter assay, gentle heating at 60°C was tested for its ability to shift the L-13 liver cytosol peak to that of the MT monomer (Fig. 1A). After heating, 90% of the recovered $^{109}$Cd was indeed associated with a peak having a $V_e/V_o$ typical of MT (Figs. 1A and 3D). A somewhat similar shift of cadmium-binding peaks in gel filtration was observed by Chan et al. (1993) after plasma samples from pregnant rats were heated to 95°C for 5 min. Similarly, when the final supernatants from the Cd/hemoglobin assays of liver (Figs. 1E and 1F), kidney (Fig. 1B), and duodenum (Fig. 1C) were separated by gel filtration, varying amounts of $^{109}$Cd were bound to higher-molecular-weight fractions. In this case, reducing the duodenum fractions ($V_e/V_o = 1.3$) with 2-ME and re-chromatographing them caused nearly all the $^{109}$Cd to be associated with a typical MT peak. Whether this type of aggregation, which has been demonstrated in vitro (Tang et al., 1999), is one that occurs or has physiological significance in vivo is not currently known.

Measurements of MT mRNA provide information about the level of control of the MT increase observed during lactation. While our results in the mouse clearly showed significantly increased MT protein levels in the liver, kidneys, and duodenum of mid-lactating mouse dams, Andersen et al. (1983) reported in the rat that hepatic MT mRNA concentrations, as measured by in vitro translation, rose sharply just prior to parturition and fell to a low level throughout lactation. Our demonstration of an abrupt fall of MT protein to levels comparable to non-pregnant controls by 5 days after weaning, as well as a corresponding drop of liver MT mRNA between L 13 and W 5 (Fig. 2), indicate that neither molecule persists long after the cessation of the stimulus of lactation in the mouse. More data are needed to reconcile these differences and determine whether there may be a species difference between mouse and rat in effects of lactation on MT protein and MT mRNA concentrations. Ours are the first results, however, to clearly demonstrate striking corresponding increases in both MT protein and MT mRNA concentrations during lactation that fall abruptly to low levels after cessation of lactation.

In summary, mid-lactating mouse dams that were free of exogenous Cd showed appreciably increased MT concentrations in the cytosols of liver, kidneys, and duodenum, but not jejunum, as measured by Cd/hemoglobin radioassays, gel filtration analyses, and, indirectly, by m-RNA analyses. This lactation-induced MT was shown to form a Cd-MT complex in the dam tissues after intestinal absorption of small amounts of cadmium during lactation. The increases in MT appeared to be induced by physiological changes associated with lactation, because post-lactational dams showed abrupt decreases in MT and in MT mRNA levels. Additional investigations are required to demonstrate whether these MT changes might play a role in pathways for handling essential metals such as copper and zinc in the dam. However, the MT changes reported here appear to explain results of previous studies showing altered cadmium uptake and tissue distribution in pregnant and lactating mice continuously exposed to very low concentrations (0.1 ppb) of cadmium. (Bhattacharyya et al., 1981, 1982, 1986; Whelton et al., 1993). In the latter mouse model, increases in cadmium intestinal absorption and tissue retention, combined with lactation-induced increases in overall diet consumption, resulted in 6- to 8-fold increases in the kidney cadmium concentrations of mouse dams by the end of lactation, compared to those of the nonpregnant controls. Because diet is the major source of environmental exposure to cadmium in humans, female humans while nursing might show analogous increases in intestinal absorption and renal accumulation of dietary cadmium, with resultant increases in the susceptibility of multiparous females to the adverse effects of long-term cadmium exposure.

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