

# **Immunohistochemical detection of metallothionein in liver, duodenum and kidney after dietary copper-overload in rats**

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**Summary.** Metallothionein (MT) has been used in immunohistochemical techniques to indicate presence and distribution of heavy metals within biological tissues. This study describes a comparison of the pattern of MT-immunostaining in the liver, duodenum and kidney during dietary copper overload in rats. Sixteen male 10-week-old Wistar rats were randomly allocated into groups of four. Two groups were fed a pelleted diet containing 1,500 mg/kg copper and two control groups received a rodent diet containing 10 mg/kg copper. After 6 weeks samples of liver, kidney and duodenum were collected for immunohistochemistry and histology. An indirect immunoperoxidase technique, using monoclonal antibody E9 against horse MT and polyclonal sera against rabbit MT, was employed. Copper-loaded rats had marked MT-immunoreactivity within the nucleus and cytoplasm of many periportal hepatocytes, renal proximal convoluted tubule epithelial cells, intestinal columnar epithelial cells and Paneth cells. Immunohistochemical staining was similar using either mouse anti-MT polyclonal serum, or monoclonal antibody E9. Hepatocytes surrounding inflammatory foci were positive for MT, supporting the proposed role of this protein in free radical scavenging. The presence of MT in the kidney appears to be associated with renal excretion of copper-metallothionein (Cu-MT) in copper-loaded rats. Paneth cells were easily detected using MT-immunostaining. MT may play a part in absorption of copper from intestinal contents and possible storage as Cu-MT in Paneth cells. The function of Paneth cells remains unknown but the presence of marked MT-immunoreactivity in these cells, observed in copper-loaded rats, suggests their involvement in homeostasis and metabolism of copper.

**Key words:** Metallothionein, Immunoperoxidase, Copper, Kidney, Paneth cells

## **Introduction**

Metallothionein (MT) is a low molecular weight (6000 D), cysteine rich, cytoplasmic protein, with a high affinity for cadmium (Cd), copper (Cu), zinc (Zn) and other heavy metals. It is found in normal animal tissues bound to Zn and Cu (Kägi and Kojima, 1987). MT is present in virtually all vertebrates and invertebrates (Hamer, 1986; Kägi and Kojima, 1987). It is thought to function in homeostasis, storage and transport of Cu and Zn (Karin, 1985; Dunn et al., 1987) and to bind to toxic metals, thus detoxifying them (Brady, 1982; Hamer, 1986). Other proposed functions include free radical scavenging (Thornally and Vasak, 1985) and a role in immune function (Cherian and Goyer, 1978).

In recent years, MT has been used in immunohistochemical techniques, to indicate presence and distribution of heavy metals, in particular Cu, within biological tissues (Williams et al., 1989; Evering et al, 1990), and as a diagnostic tool for human patients with Cu-associated diseases (Janssens et al., 1984; Elmes et al., 1989). However, a comparison of the distribution of immunoreactive MT in the liver, intestine and kidney during dietary copper overload has not been described in the literature.

The purpose of this study was to enhance our knowledge of the role of MT in Cu toxicity and tolerance in copper-loaded rat liver, kidney and small intestine, using an indirect immunoperoxidase technique.

## **Materials and methods**

### *Experimental animals*

All animals used in this study were housed and cared for according to Canadian Council of Animal Care guidelines. Sixteen male 10-week-old Wistar rats (Charles River, St. Constant, Quebec), were randomly placed in groups of four. Two groups were fed rodent laboratory chow (Purina, St. Louis, MO) containing 1,500 mg/kg CuSO<sub>4</sub> (Teklad Diets, Madison, WI) and two groups were fed rat chow without the Cu

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supplement (<10 mg/kg Cu). Rats were maintained in an environment of 12-hour light and dark cycles. Food and water were given free choice. After 6 weeks rats were placed in a carbon dioxide chamber until they became unconscious and then killed by cervical dislocation. Samples of kidney cortex, liver right medial lobe and duodenum were removed from each rat and fixed for 24 hours in 10% neutral buffered formalin.

### *Preparation of metallothionein and immunization*

Rabbit MT (Sigma Chemical co., St. Louis, MO) was purified by anion exchange performed using fast protein liquid chromatography (Pharmacia, Montreal) as described elsewhere (Olsson and Hogstrand, 1987).

Purified MT was polymerized into subtle complexes with glutaraldehyde (Garvey et al., 1982) and then mixed with Ribi's adjuvant (Sigma) in a 1:1 ratio (v/v). Three 15-16 week old Balb/c mice (Charles River) were injected intraperitoneally with 100 µg of polymerized MT on days 0, 21, 35, 45 and 55. The final injection did not contain Ribi's adjuvant. The mice were anaesthetized with halothane (Wyeth-Ayerst, Montreal), and exsanguinated by transthoracic cardiac puncture. Serum was stored at -20 °C.

### *Histology*

Tissue sections were dehydrated through an ascending series of graded ethanol, cleared in xylene, embedded in paraffin, sectioned at 5 µm thickness and stained with haematoxylin and eosin.

### *Immunohistochemistry*

Tissue sections were applied to 0.1% w/v poly-L-lysine coated slides (Sigma). All reagents were prepared in Dulbecco's phosphate buffered saline (D-PBS) and used at room temperature. Tissues were deparaffinized, hydrated and treated sequentially with 1.5% hydrogen peroxidase for 30 minutes, 1% normal goat serum (Vector Lab Inc., Burlingame, CA) for 30 minutes and primary antibody for 60 minutes. Polyclonal sera against rabbit MT diluted 1:400 or monoclonal antibody E9 against horse MT at 1:12000 (a kind gift from A. Cryer, J. Kay and J.M. Stark, University of Wales College of Medicine, Cardiff, UK) were used. Next, slides were treated with horseradish peroxidase-goat anti-mouse IgG (Cappel, Organon Teknika Corporation, Durham, NC) at 1:200 for 60 minutes and substrate for 4 minutes. Substrates used consisted of 18 mL D-PBS, 7 µL of 30% hydrogen peroxide and 10 mg 3,3'-diaminobenzidine (Sigma). Between each step, the slides were washed in D-PBS/0.1% Tween-20 for 10 minutes. Slides were counterstained with aqueous 4.7% haematoxylin (Shandon, Pittsburgh, PA) for 5 seconds, dehydrated in a graded ethanol series and cleared with xylene. Slides were mounted with Flo-texx (Lerner Laboratories, Pittsburgh, PA) and examined with a Zeiss light

microscope.

Specificity of the staining reaction was assessed in several different control experiments: prior absorption of primary antibody with rabbit MT, substitution of primary antibody with D-PBS or normal mouse serum (1:100), and omission of both primary and secondary antibodies.

## **Results**

### *Histology*

In copper loaded rats, liver sections had multifocal accumulations of neutrophils and macrophages associated with necrotic foci. Numerous degenerating hepatocytes, characterized by hypereosinophilic cytoplasm and pyknotic nuclei, were seen. There was a moderate inflammatory response around zone 1 (periportal areas) consisting predominantly of lymphocytes with a few macrophages. Many cells in the proximal convoluted tubules (PCT) contained intracytoplasmic and intra-luminal hyaline droplets. Histological changes were not detected in the duodenum.

Lesions were not observed in the liver, kidney and duodenum from control rats.

### *Immunohistochemistry*

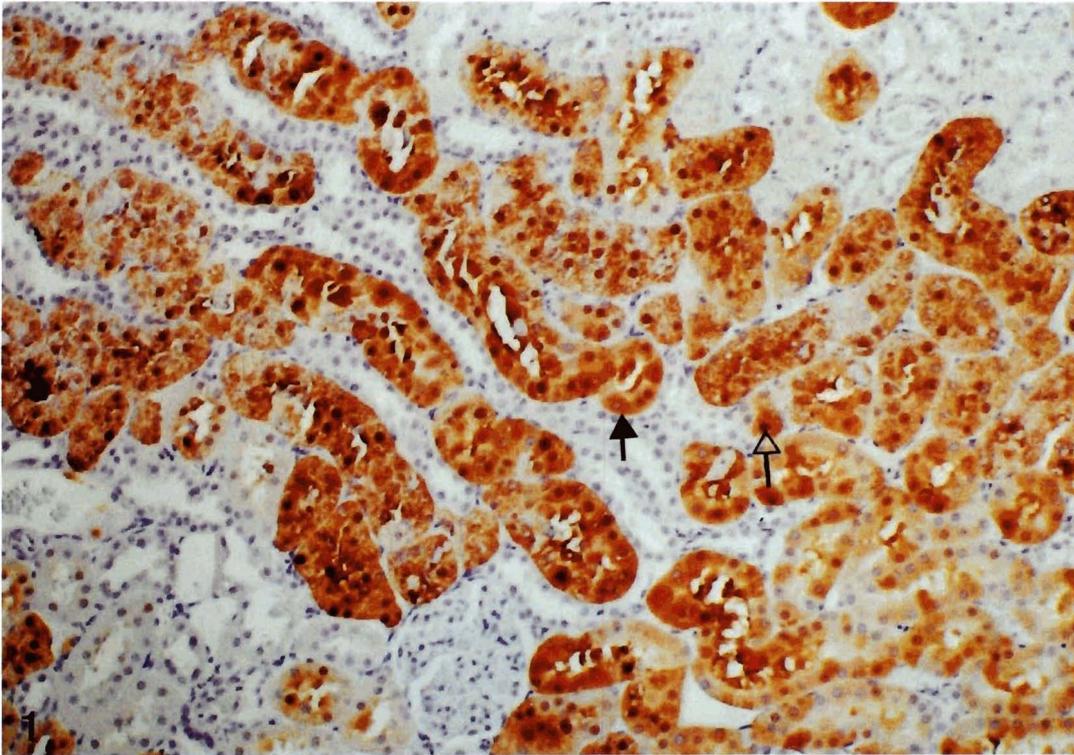
The immunohistochemical reaction using either mouse anti-MT polyclonal serum, or E9 showed specific staining for MT. Staining was not detected in negative controls. The absorption of mouse polyclonal anti-MT or E9 with rabbit MT abolished staining. Also, application of normal mouse serum and D-PBS did not result in staining. In both copper-loaded and control rats all findings were similar whether sections were stained with monoclonal antibody E9 against horse MT diluted 1:12000 or polyclonal sera against rabbit MT at 1:400.

In copper-loaded rats, moderate to marked immunoreactivity for MT was present in the cytoplasm of most hepatocytes. Staining was variable between liver zones (1, 2 and 3), and between individual cells with the nuclei and cytoplasm of liver cells in zones 1 and 2 staining most intensely. Inflammatory foci were surrounded by markedly immunoreactive hepatocytes. Staining was not observed in vascular endothelial cells and bile duct epithelium.

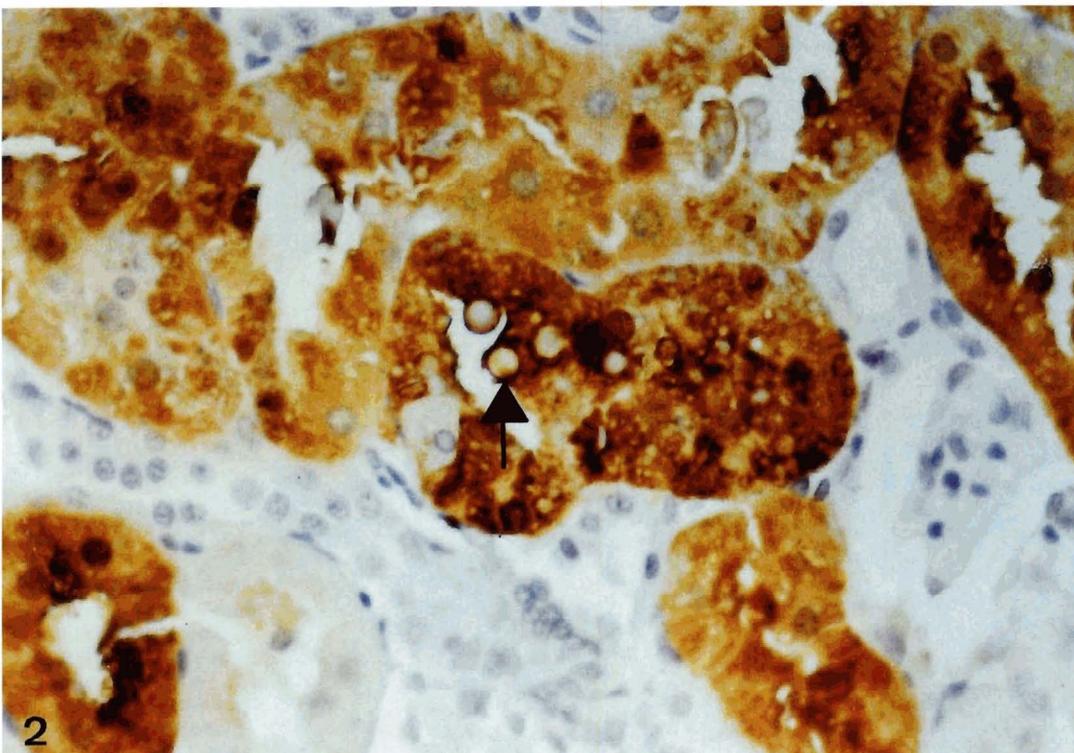
Marked immunoreactivity for MT was seen in both cytoplasm and nuclei of renal cortical PCT cells of copper-loaded rats (Fig. 1). Hyaline droplets within PCT cells did not stain for MT (Fig. 2). Staining was segmentally distributed in rays extending from the capsular surface toward the medulla. Adjacent to strongly positive regions were non-staining regions. Moderate staining was detected in the cytoplasm and nuclei of occasional cells in the distal convoluted tubules (DCT). Staining was absent in renal glomeruli and vascular smooth muscle cells.

Immunoreactivity for MT was seen in specific cells in the small intestine of copper-loaded rats. Villous

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**Fig. 1.** Copper-loaded rat kidney. Immunostaining of proximal convoluted tubules (cytoplasm: closed arrow; nucleus: open arrow). E9 against horse metallothionein and haematoxylin counterstain. x 176



**Fig. 2.** Copper-loaded rat kidney. Negative immunostaining of hyaline droplets (arrow) within proximal convoluted tubule epithelial cells. E9 against horse metallothionein and haematoxylin counterstain. x 440

columnar epithelium displayed moderate to intense MT staining in cytoplasm and nuclei. The cytoplasm of Paneth cell at the base of the crypts of Lieberkühn stained strongly positive for MT (Fig. 3). Goblet cells, the lamina propria, submucosa and serosa of the small intestine did not stain.

Immunostaining was seen in a few scattered cells in the liver, kidney and intestine from control rats. Occasional hepatocytes from control rats had faint diffuse cytoplasmic and nuclear staining. Weak immunoreactivity for MT was seen in the nuclei and cytoplasm of a few cells in the PCT. As in copper-loaded rats, staining had a segmental distribution extending in rays from the outer cortex toward the medulla. Sporadic weak immunostaining was seen in the cytoplasm of cells in the DCT, and in the collecting ducts. Immunostaining was rarely detected in Paneth cells in control rats.

### Discussion

Prominent MT immunoreactivity was detected by immunoperoxidase staining in Cu-loaded rat liver, duodenum and kidney. Conversely, weak immunostaining was seen in tissues of control rats. These findings add support to the proposed role of MT in cellular detoxification of Cu and other trace elements (Cousins, 1985; Evering et al., 1990; Freedman and Peisach, 1989; Fuentealba et al., 1993).

The metabolism of Cu in the liver may occur through three possible paths: the metal is incorporated into

ceruloplasmin, stored in lysosomes or Cu-binding proteins such as MT, or it may be excreted into the bile (Cousins, 1985). Metallothionein synthesis, evidenced by marked immunostaining, occurred predominantly in periportal areas (zone 1) in Cu-loaded rats. Periportal MT-immunoreactivity has been described in other studies using a variety of immunohistochemical methods (Banerjee et al., 1982; Danielson et al., 1982; Williams et al., 1989; Evering et al., 1990). This preferential distribution of MT supports the concept of microheterogeneity of liver function in which hepatocytes in different zones are thought to differ in enzymic and subcellular structures (Jungermann and Katz, 1989).

Multifocal areas of necrosis surrounded by lymphocytes and macrophages and lympho-plasmacytic periportal inflammatory infiltrations were seen in the livers of copper-loaded rats. The intense MT-immunostaining around these inflammatory foci in Cu is particularly interesting. MT has been shown to be an efficient free-radical scavenger *in vitro* (Thornally and Vasak, 1985). Free radicals released during the inflammatory response are scavenged by MT in an attempt to protect the tissues from further damage, hence, increased levels of MT and in turn, increased intensity of immunostaining. Another possible explanation is that condensation of cellular debris could result in more intense immunostaining than would be seen normally. Narthey et al. (1987) in a study of human patients with Wilson's disease also found intense cytoplasmic and nuclear staining where extensive hepatocellular degeneration had occurred. The

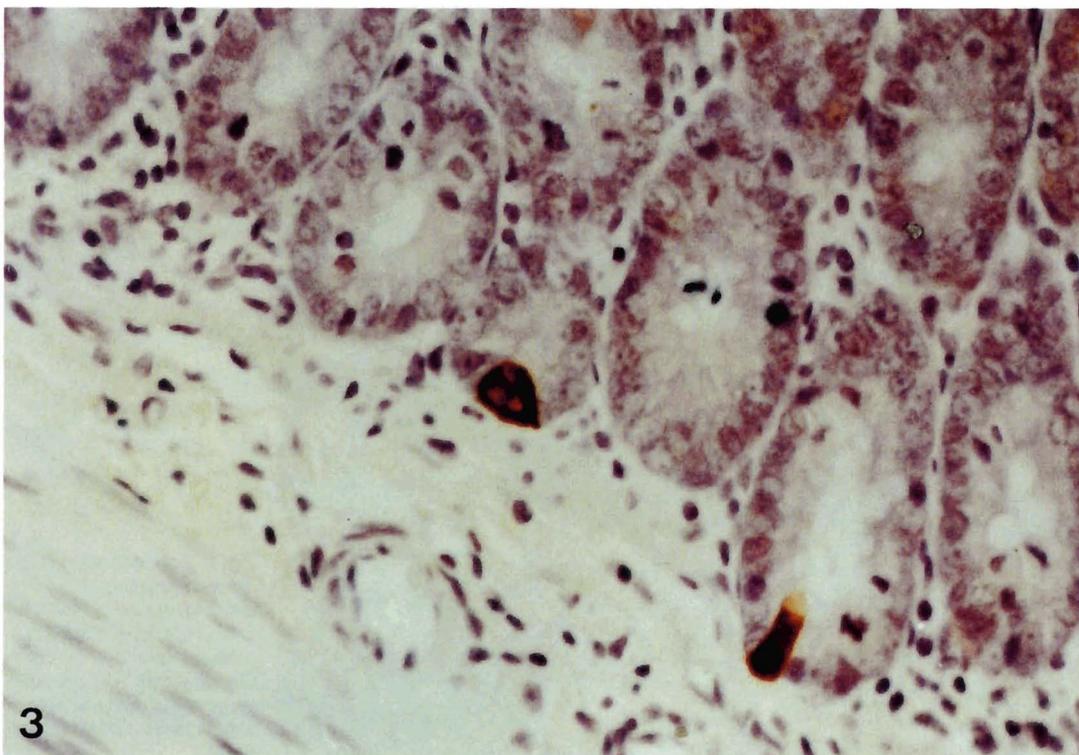


Fig. 3. Section of small intestine from a copper-loaded rat. Paneth cells stain strongly positive with polyclonal serum against rabbit metallothionein. Haematoxylin counterstain. x 440

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presence of high levels of MT in necrotic hepatocytes also suggest that elevated Cu-MT content are toxic to the liver cell (Fuentelba et al., 1993). Microanalytical studies in copper-loaded rat hepatocytes have demonstrated that in addition to copper, the nucleus and lysosomes contain high levels of sulphur (Fuentelba et al., 1989a,b). This finding provided indirect evidence of the presence of MT, a sulphur rich protein, in the liver of Cu-loaded rats.

In rats used in this study, the hepatic Cu content was  $581 \pm 182 \mu/\text{gr}$  (wet tissue weight) in the copper-loaded group and  $4.2 \pm 0.2 \mu/\text{gr}$  in control rats (Mullins, 1995). Therefore, although the histological lesions observed in the copper-loaded rats are significant, they do not reflect the magnitude of hepatic Cu accumulation. A possible explanation for the lack of correlation between microscopic lesions and high levels of hepatic Cu is the development of tolerance described in male Wistar rats exposed to high Cu levels (Haywood, 1985; Fuentelba et al. 1989a,b, 1993; Fuentelba and Bratton, 1994). The mechanism of resistance to the toxic effects of copper has been investigated using Cu-resistant hepatoma cells by Freedman and Peisach (1989). These authors demonstrated that the level of metal resistance is proportional to the concentration of Cu-MT. Therefore, tolerance in copper-loaded rats may be related to a variety of factors including MT synthesis, Cu sequestration within lysosomes and/or nuclear sequestration of Cu (Fuentelba et al., 1993).

Haywood (1985) suggested that Cu-MT in the proximal tubular cells prevents the reabsorption of copper from the glomerular filtrate. In the present study, marked immunoreactivity for MT was identified in PCTs and some collecting tubules and DCTs in copper-loaded rats. The staining was especially pronounced in the outer cortex, and in segmental extensions into the medulla. The predominance of MT immunoreactivity in the outer cortex may represent a zonal distribution of cells which have prior commitment to MT synthesis such as exists in the liver (Evering et al, 1990). The pattern of segmental distribution of MT may also reflect vascular supply which in turn can influence cellular metabolism.

Increased numbers of hyaline droplets in PCT cytoplasm and lumina have been previously identified in the kidneys of Cu-loaded rats (Haywood, 1985; Fuentelba et al., 1989b; Evering et al., 1990). The hyaline droplets are composed of alpha-2-urinary globulin, a protein that is synthesized in the liver and excreted through the kidneys (Alden, 1986). The droplets have been associated with nephropathy (Neuhaus, 1980) and with excess renal Cu accumulation (Haywood, 1985; Fuentelba et al., 1989b). The presence of Cu within hyaline droplets has been confirmed using histochemical (Haywood, 1985) and microanalytical techniques (Fuentelba et al., 1989b). Increased numbers of droplets containing Cu in the lumina of some PCT supports an earlier study suggesting extraluminal excretion of Cu (Haywood, 1985). Therefore, the kidney can contribute to Cu homeostasis

using two mechanisms, sequestration of Cu bound to MT and excretion of the metal as Cu-MT or via hyaline droplets.

The role of the small intestine in copper toxicity and tolerance has not been clearly elucidated. However, it has been previously demonstrated that the duodenum accumulates copper as a result of excess dietary intake of the metal (Fuentelba and Bratton, 1994). Specifically, in rats receiving a diet containing 1,500 mg/kg Cu for a period of 5 weeks the copper content of the duodenum was  $134 \mu/\text{gr}$  (wet weight) compared to  $5 \mu/\text{gr}$  (wet weight) in control rats (Fuentelba and Bratton, 1994). The same study demonstrated the capacity of the rat duodenum to unload copper after situations of excess dietary copper which are followed by a period of normal diet. Conversely, the gastrointestinal excretion of copper in patients with Wilson's disease is significantly lower than normal persons (Strickland et al., 1972). MT may play an important role in the intestinal absorption and sequestration of trace elements. It has been suggested that MT-rich enterocytes inhibit absorption of copper from the intestinal lumen (Mills, 1980). Therefore, in the copper-loaded rat MT synthesis in intestinal epithelial cells seems to be upregulated in response to increase Cu levels. This hypothesis is supported by the intense immunoreactivity of the cytoplasm of villous columnar epithelial cells in Cu-loaded rats and the absence of immunoreactivity in control rats. Similar findings were reported in Cu-loaded rats using anti-MT antibodies and a DNP-linked peroxidase system (Hair-Bejo et al., 1990), and in Cd-injected rats using an immunoperoxidase method (Danielson et al., 1982).

Paneth cells at the base of the crypts of Lieberkühn in the duodenum were strongly immunoreactive for MT in Cu-loaded rats. The significance of Paneth cell staining is not clearly understood since the function of Paneth cells is not yet elucidated. These cells have morphologic features characteristic of cells that synthesize and secrete proteins, such as large amounts of well-organized rough endoplasmic reticulum, a well developed Golgi apparatus and prominent secretory granules in the apical cytoplasm (Ross et al., 1989). It has also been reported that these cells accumulate heavy metals (Danielson et al., 1982). Therefore, it is reasonable to assume that Paneth cells are involved in storage, elimination and homeostasis of Cu.

Presence of MT in nuclei is characteristic of a neonatal morphological pattern due to its occurrence in the nuclei of fetal and neonatal rats (Elmes et al., 1991). The biological presence of MT in cellular nuclei is not clearly understood but is thought to be indicative of MT synthesis (Banerjee et al., 1982; Danielson et al, 1982; Williams et al., 1989). Others have suggested that metals may bind directly to chromosomes within the nucleus thereby causing expression of the MT gene, and subsequent protein synthesis in the cytoplasm (Bryan and Hidalgo, 1976). Thus nuclei of Cu-loaded rats stain strongly, since hepatocytes are more likely to be actively

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synthesizing MT to sequester Cu than control rats.

Histological lesions were not observed in control rats and weak MT-immunoreactivity was diffusely localized in the hepatocyte cytoplasm, renal PCT cell cytoplasm and some nuclei, and Paneth cell cytoplasm. Other studies evaluating immunohistochemical techniques have found MT in control rats. Polyclonal antibodies to MT demonstrated immunoreactivity in the cytoplasm (Banerjee et al., 1982), and the nuclei and cytoplasm of periportal hepatocytes (Williams et al., 1989), whereas the use of monoclonal antibody E9 detected MT-immunoreactivity in the periacinar zone (zone 3) of control rats (Evering et al., 1990). MT was found in renal collecting duct and DCT epithelium (Banerjee et al., 1982), or renal collecting duct and PCT epithelium (Williams et al., 1989), or PCT epithelium alone (Evering et al., 1990). MT was absent from glomeruli, vascular endothelium and smooth muscle cells, as evaluated by immunoperoxidase technique in this study. This is in agreement with some studies (Danielson et al., 1982; Evering et al 1990) and is disputed by others (Banerjee et al., 1982; Williams et al., 1989). The difference in staining localization between studies is unknown. However, it may represent the use of different antibodies and/or different techniques. Different antibodies may have different affinities for MT bound to different heavy metals due to changes in conformation of the antibodies (Suzuki and Sato, 1995). Techniques vary in a multitude of factors: including antibodies, reagents, and time of steps, all of which influence the intensity of staining.

The results of this study suggest that MT plays an important role in experimentally induced Cu toxicity in several organs. The presence of intense MT immunoreactivity in hepatocytes adjacent to inflammatory foci indicates either a response to increased levels of Cu, or an indirect response to elevated hepatic copper content mediated by inflammatory stimuli. MT probably plays a role in intestinal Cu absorption and in turn Cu sequestration. This hypothesis is supported by the presence of strong MT-immunoreactivity in the epithelium of the small intestine and Paneth cells.

Paneth cells appear to provide long term storage and secretion of Cu thereby contributing to Cu homeostasis. The exact nature of the role of Paneth cells in Cu toxicity and tolerance is still unknown and warrants further investigation.

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