ZINC METABOLISM IN HEMATOPOIETIC SYSTEMS

By

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For my family
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<td>rhIL-1α</td>
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<td>TNF</td>
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ZINC METABOLISM IN HEMATOPOIETIC SYSTEMS

By
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Chairman: Dr. Robert J. Cousins
Major Department: Food Science and Human Nutrition

Zinc metabolism and metallothionein expression in rat bone marrow were investigated under conditions of varying zinc status, cytokine administration and ionizing radiation injury. Roles for zinc and metallothionein during erythrocyte maturation were also investigated. These are the first studies to measure metallothionein and examine zinc metabolism in stem cell progenitors in the marrow.

Chromatographic separation of marrow cytosol, indirect protein assay and Northern blot analysis of metallothionein mRNA confirmed the presence of this protein in the marrow. Dietary zinc restriction demonstrated marrow susceptibility to zinc depletion, but no accumulation with supplemental zinc intake. Kinetic studies, following induced erythropoiesis in zinc depleted rats, showed increased zinc uptake by the marrow. Zinc increased marrow metallothionein levels 2-fold, while dexamethasone decreased levels. Long-
term exposure to high levels of zinc by mini-osmotic pump did not continue to increase marrow metallothionein levels. These data suggest that marrow zinc concentrations are tightly regulated and correlate to tissue metallothionein levels.

Interleukin-1, interleukin-6 and ionizing radiation were used to initiate the acute-phase response and tissue injury in rats of varying zinc status. Although a significant interaction between zinc status and cytokine administration was measured in the liver, no interaction was measured in the marrow. Metallothionein-1 is the predominant isoform expressed by the marrow. Decreased metallothionein was measured above 0.5 Gy radiation.

Administration of zinc to rats made anemic by phenylhydrazine treatment led to an increase in metallothionein, compared to anemic control rats. When this marrow was separated over a standard discontinuous Percoll gradient, proportionally more metallothionein was measured in the erythroblast fraction.

K562 cells accumulate zinc and metallothionein in response to increasing medium zinc concentrations, but not to interleukin-1 nor interleukin-6. Hemin induced differentiation decreased metallothionein, regardless of zinc concentration. When hemin and zinc were provided simultaneously, metallothionein mRNA was not different from non-hemin treated control cells. Incubation with mitomycin-
C eliminated metallothionein induction by 100 μM zinc, suggesting that metallothionein accumulation occurs in proliferating cells. Lower metallothionein levels would be expected if zinc is being released from the protein and/or if less zinc is available to initiate metallothionein gene transcription.
CHAPTER 1
INTRODUCTION

First identified in 1960 by Kagi and Vallee, the metal-binding protein, metallothionein, has been characterized both physicochemically and physiologically. Areas of current interest are the roles of zinc and metallothionein in cellular immunity, both as cytoprotective agents and in the differentiation or clonal expansion of hematopoietic stem cells. However, zinc metabolism in bone marrow progenitor cells is unexplored.

Huber and Cousins (1988) characterized the effects of interleukin 1 (IL-1) on metallothionein and zinc metabolism in late gestation fetal rat liver. At this stage of development, the liver is the primary hematopoietic organ of the animal. In addition, zinc kinetics data show a transient accumulation of zinc in the bone marrow of rats administered interleukin-1 (Cousins and Leinart, 1988; Huber and Cousins, 1988). Other workers have reported increased metallothionein in liver following irradiation, and a protective effect with pre-radiation zinc treatment (Floersheim et al., 1988, Matsubara et al., 1988, Shiraishi et al., 1989). Similar effects were observed in mice pretreated with interleukin-1 (Schwartz et al., 1987; Neta
et al., 1988 and Dorie et al., 1989). These experiments are all suggestive of a protective role for metallothionein.

Additional data suggest that the population of hematopoietic cells which respond to increased zinc concentrations by altering metallothionein expression are immature erythrocytes. Tanaka et al. (1985) described increased metallothionein concentrations in red blood cell lysates from rats 2-4 days following administration of large doses of CdCl₂. This time frame correlates well with the time required for reticulocyte maturation in the marrow and release into the circulation. Similarly, metallothionein increased within 3-5 days in red blood cell lysates from human subjects supplemented with 50 mg zinc gluconate/d, and rapidly declined upon cessation of supplementation (Grider et al., 1990). Additionally, Robertson et al. (1989) have shown increased metallothionein-1 in the youngest reticulocyte fractions in response to changes in dietary zinc intake.

Together these data suggest there is a potential influence of zinc status on the differentiation of stem cell progenitors and/or their metabolic parameters. Zinc is an essential trace element whose broad spectrum of actions has been well described. The bone marrow represents a metabolically active organ which produces billions of cells per day, yet must be able to up-regulate the constitutive level of hematopoiesis to defend against infectious insults
and tissue injuries. Increased metallothionein in this tissue may function as a labile source of necessary zinc and/or could serve a cytoprotective role.

The objective of this study was to determine whether changes in metallothionein expression reflected altered zinc metabolism in hematopoietic systems. Investigations were conducted in the bone marrow of intact rats and at the cellular level in a representative cell type, the K562 erythroleukemic cell line.

These studies are separated into four chapters, each designed to answer specific questions. The first section (Chapter 4) discusses general observations about zinc metabolism in the bone marrow. These experiments were designed to discover whether metallothionein was present in the bone marrow and whether it could be altered with dietary zinc intake.

The next group of experiments (Chapter 5) investigates some aspects of stress and their relationship to zinc metabolism and metallothionein expression. The first question investigated was whether metallothionein concentrations in the bone marrow changed in response to the cytokines, interleukin-1 and interleukin-6, agents known to orchestrate many metabolic and hematological changes associated with host defense mechanisms. Secondly, pre-treatment with both zinc and interleukin-1 has been shown provide a protective effect against irradiation exposure.
It was investigated whether marrow metallothionein is altered in response to ionizing radiation exposure.

The third group of experiments (Chapter 6) investigated changes in zinc and metallothionein within a specific sub-population of marrow cells: erythrocytes. Several experiments were designed as a corollary to the human zinc supplementation work indicating that the metallothionein concentration may be an accurate marker for zinc nutriture. Evidence was gathered to substantiate that metallothionein synthesis takes place in the pronormoblasts present in the marrow.

Finally, these findings were examined on the cellular level using the K562 erythroleukemic cell line, and are discussed in Chapter 7. The main interest in these experiments was the response of the cells to increased zinc exposure and changes in metallothionein expression which could be linked to differentiation and proliferation of the cells.

The final chapter is an integration of the results of all these studies, leading to an overall concept of zinc metabolism and metallothionein expression in the bone marrow, and specifically the erythrocytic cells. There is some discussion of technical difficulties and suggestions for future investigations in this area.
CHAPTER 2
REVIEW OF THE LITERATURE

Chemistry of Zinc

Although zinc was identified as an essential element for the growth of *Aspergillus niger* by Raulin in 1896 (Spiro, 1983), its critical importance in mammalian species was not appreciated until the 1930s (Todd et al., 1934). Zinc deficiency in human populations was not recognized until the early 1960s in the Middle East (Prasad, 1963). The advent of analytical methods to measure zinc, atomic absorption spectroscopy, analysis by isomorphous substitution, and the use of radioisotopes have been instrumental in defining the role of zinc in metabolism.

Zinc is implicated in alterations in cellular replication, bone formation, skin integrity, cellular immunity and fetal development. These physiological functions of zinc have frequently been related to acrodermatitis enteropathica (a genetic disorder of zinc metabolism) and its occurrence in the structure of many metalloenzymes (Hambidge et al., 1986).

With only broad roles for zinc defined, it is useful to consider the physical and chemical properties of the element that would support such roles. Zinc is a IIB metal of the
first transition series with a full d\(^{10}\) subshell, making it diamagnetic and not susceptible to ligand field stabilization interactions. The stereochemistry of zinc binding is determined by the electrostatic and covalent binding forces and by ligand size. Zn(II) prefers tetrahedral complexes with a flexible co-ordination geometry, with acidic complexes of co-ordination numbers 4 or 5 being most commonly encountered. Furthermore, zinc maintains a single oxidation state and does not participate in redox chemistry, unlike the essential series IB transition metals (i.e. Cu and Fe), thus making it an ideal structural molecule (Vallee and Galdes, 1984).

Zinc has been identified as a constituent of 202 enzymes from various species, participating in reactions of polymerases, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Vallee and Galdes, 1984). When one corrects this number for observations of the same enzyme in more than one species, the number is still at least 60 different enzymes (Cousins and Hempe, 1990). Its roles have been characterized as either catalytic, structural, regulatory or non-catalytic. For example, the red blood cell enzyme, carbonic anhydrase, utilizes its lone zinc atom in a catalytic capacity. Chelation of the metal does not alter the tertiary protein structure, but does eliminate enzyme activity (Lindskog and Malmstrom, 1960).
With respect to its involvement in the catalytic activity of metalloenzymes, the constituent Zn\(^{2+}\) atoms are often buried deep within the hydrophobic multidentate regions of enzymes, rather than binding to charged residues on the protein surface, like Ca\(^{2+}\) or Mg\(^{2+}\), which function to trigger rapid conformational changes in the protein (Williams, 1981). Instead, zinc participates as an excellent Lewis acid in hydrolytic reactions. The unique interaction between zinc and water also makes it important for the catalysis of CO\(_2\) and the formation and catalysis of phosphate esters critical in nucleic acid chemistry (Williams, 1981).

Most often, however, zinc functions in a structural capacity, by maintaining quartenary structure of an enzyme complex, but not participating in the overall reaction. Removal of zinc leads to a disruption of activity through loss of protein conformation. Zinc mediates a regulatory role in fructose-1,6-bisphosphatase, inhibiting enzymatic function, rather than catalyzing the reaction or aiding in the maintenance of structure (Tejwani, 1983).

The non-catalytic role for zinc in metalloenzymes describes a group of enzymes which contain zinc, but its purpose is undefined. For example, alcohol dehydrogenase has one zinc atom in a catalytic site, and another zinc atom of unknown function, the so called non-catalytic zinc atom.
Non-enzymatic Biological Functions for Zinc

Elaborate mechanisms describing the action of zinc in biological systems remain speculative. The fragility of the erythrocyte membrane from zinc-deficient animals suggests that zinc acts to alter membrane characteristics, providing a useful hypothesis to explain many symptoms associated with the zinc deficiency syndrome (Bettger and O'Dell, 1981). Additionally, through many phospholipid or thiol group linkages, zinc may act to stabilize membranes altering receptor binding sites at the cell membrane. This may affect internalization kinetics and intracellular signalling mechanisms (Csermely et al., 1989). However, the effect of zinc concentrations on membrane fluidity and stability varies with cell type (Chvapil et al., 1976, Weston et al., 1977, Fenton et al., 1980, Bettger and O'Dell, 1981).

Erythrocytes obtained from healthy human subjects on zinc supplements showed increased metallothionein and zinc levels within 10 days of initiating supplementation (Grider, et al., 1990). With time the levels reached a plateau, then declined rapidly when supplementation was withdrawn. It is hypothesized that the high level of erythrocyte metallothionein occurs in the young reticulocytes and is not maintained throughout red cell aging. Similar changes in red blood cell metallothionein levels were measured in iron deficient anemic rats, with highest metallothionein
concentrations found in the young reticulocyte fractions (Robertson et al., 1989).

Metallothionein in the red blood cells of mice was suggested to be synthesized by the reticulocytes and released into the circulation as the cells mature in studies of cadmium-induced metallothionein (Tanaka et al., 1985). Particularly high levels were encountered during active erythropoiesis and the protein disappeared from the red blood cells according to the life span of the red blood cell (Tanaka et al., 1986). These data suggest that metallothionein may function early in red cell maturation in a capacity other than membrane stabilization, but ceases to be important after the cell reaches terminal differentiation.

Specialized cell functions have been shown to be impaired by either high or low levels of zinc in several studies, but a causative mechanism has yet to be defined. For example, macrophages cultured from guinea pigs maintained on a high zinc diet were functionally immobilized and exhibited decreased phagocytosis, compared to cells from guinea pigs fed two diets containing less zinc (Chvapil et al., 1976). This phenomenon was reversed by reincubating the cells in zinc-free medium, eliminating the possibility that this was an effect of zinc toxicity. Platelet aggregation was shown to be inhibited when a supplemental level of zinc was present in the medium in the same study.
Other groups defined a very narrow range of zinc concentration (0.1-0.2 mM) which was able to induce 10-40% of lymphocytes to transform into lymphoblasts within 6-7 days (Ruhl et al., 1971, Berger and Skinner, 1974).

Neutrophils have 6- to 8-fold greater zinc concentrations than the plasma, and are very effective at sequestering zinc during infection (Beisel, 1977). In contrast to data from macrophages, chemotaxis by neutrophils, cultured from patients with acrodermatitis enteropathica, improved in a linear dose-dependent fashion when incubated with zinc (Weston et al., 1977). Additionally, chemotaxis was impaired in neutrophils cultured from zinc deficient mice compared to neutrophils from zinc-adequate control mice (Fenton et al., 1980). Subsequent investigations by the same group support the hypothesis that zinc deficiency alters the activity of membrane bound proteins, leading to the observed decrease in function. Zinc deficient mice showed lower specific activity of myeloperoxidase, an enzyme involved in the oxygen-dependent bactericidal activity, and alkaline phosphatase in the neutrophils compared to both ad libitum and pair-fed zinc-adequate control mice. However, neither oxygen consumption nor the activities of other known zinc dependent enzymes were different between the groups.

Another plausible biological mechanism with growing experimental support is that zinc influences second
messenger intracellular signalling. Zinc increases the activity of the protein kinase C (PKC) pathway in Ca²⁺ or antigen-induced T-lymphocytes, leading to increased PKC binding and a shift in intracellular zinc distribution from the cytosol to the microsomes (Csermely et al., 1987). These findings have been confirmed (Zalewski et al., 1990). The increase in PKC activity was determined to be through increased receptor number, rather than increased receptor affinity (as is the case for Ca⁺² induced changes) and was concomitant to increased zinc uptake (Forbes et al., 1989, 1990). These authors suggest that zinc may bind to the C1 domain of the regulatory protein, while calcium binds at the C2 domain. The involvement of cysteine-rich domains, which could be zinc binding sites, was also demonstrated in these proteins (Ahmed et al., 1991).

Dunn and Cousins (1988) have described changes in whole-body zinc distribution in rats following dibutyryl cAMP, suggesting a possible role for zinc in the well-described cAMP second messenger cascade. Shirakawa et al. (1988) have data supporting a role for cAMP as the intracellular signal for interleukin-1. More recently, the increase of cAMP following receipt of the interleukin-1 signal has been linked to phosphorylation by protein kinase A (Chedid and Mizel, 1990).

Zinc may become important in activation of resting T-lymphocytes either in the capacity of a second messenger, as
described for PKC or in the cAMP cascade, or through stabilization/facilitation of cytokine-receptor binding and/or internalization. In order for T-cells to become activated, two events must occur: a processed antigen must be presented in the appropriate configuration by a macrophage or B-cell and an interleukin-1 signal must be received by the resting T-cell (Dinarello and Meir, 1987). Antigen binding to the cell initiates an intracellular signal, resulting in a conformational change in a G-protein, causing phospholipase C to activate protein kinase C, which allows intracellular Ca\(^{2+}\) levels to increase (Miyajima et al., 1988). A family of cell surface receptors, including those for some cytokines, possessing a binding site for the zinc ion have been described, which supports a role for zinc in receptor/ligand binding (Cunningham et al., 1990).

Activated T-cells begin the production of a flurry of cytokines, including IL-2, IL-3, IL-6, and GM-CSF (granulocyte-macrophage colony stimulating factor). Interleukin-2 drives the clonal expansion of activated T-cells (Granelli-Piperno et al., 1984). Interleukin-3 and GM-CSF effect stem cell proliferation in the bone marrow (Hunt et al., 1987). Interleukin-6 is responsible for some of the acute phase response, including metallothionein production in the liver (Schroeder and Cousins, 1990). Several acute-phase reactant proteins contain genetic sequences with significant homology to the metal regulatory
element defined for metallothionein, suggesting another mechanism whereby zinc may influence the effects of these cytokines.

The discovery of "zinc fingers" in DNA binding proteins (transcription factors) by Klug and Rhodes (1987) reaffirms the importance of zinc for cell replication, growth, and differentiation and is another potential site where increased amounts of zinc may be needed for cells. The antagonistic effect of zinc deficiency during gestational and perinatal development supports a significant role for the metal in these processes (Anderson et al., 1983; Huber and Cousins, 1988; Panemangalore et al., 1983; Vruwink et al., 1988), as well as its requirement by DNA and RNA polymerases (Wu and Wu, 1987).

It can be hypothesized that metallothionein is either the source of the metal for the polymerases, or it performs a specific transmissional function in DNA replication and/or cellular differentiation. Zn\(^{2+}\) is bound both to metallothionein and to transcription factors in a tetrahedral formation with 4 cysteine, or 2 cysteine and 2 histidine residues. This "zinc finger" motif found in transcription factors is specific for Zn\(^{2+}\) and appears to be critical in maintaining conformational structure, rather than catalytic activity (Klug and Rhodes, 1987).

It has been proposed that the thionein/metallothionein couple may act in concert to down-regulate a number of zinc-
responsive genes (Zeng et al., 1991b). These authors present data showing increased amounts of thionein (apometallothionein) can remove the zinc from transcription factor III-A (Zeng et al., 1991b) and the Sp-1 transcription factor (Zeng et al., 1991a). However, metallothionein would not donate zinc to a zinc-depleted Sp-1 protein. Changes in metallothionein concentrations during specific cell-cycle phases tend to support such a role.

**Zinc Homeostasis**

Zinc homeostasis is modulated by alterations in absorption, excretion, and distribution of the metal between various body compartments. Recent detailed discussions of normal zinc metabolism are available (Chesters, 1982 and Cousins, 1985). Nevertheless, it is helpful to recall that the recommended daily requirement for zinc in normal healthy adults has been set at 15 mg/d for males and 12 mg/d for females (National Academy of Science, 1990), but increases during pregnancy and may increase during conditions of stress, including illness. There are many data to suggest that some changes in zinc metabolism (e.g. up-regulation of metallothionein transcription) are affected at the molecular level by hormonal and dietary signals which provide a regulatory system for zinc, without need to rely on dietary intake (Cousins, 1985).
Metallothioneins are cytosolic proteins (6 kDa) which have been implicated in the regulation of essential (Zn\(^{2+}\) and Cu\(^{2+}\)) and toxic (Cd\(^{2+}\) and Hg\(^{2+}\)) heavy metal metabolism. The physical characteristics and physiologic aspects of this protein family have been widely studied with respect to zinc and copper metabolism and cadmium detoxification (reviewed by Dunn et al., 1987; Hamer, 1986; Cousins, 1985; and Webb, 1979). Although cysteine is the predominant amino acid residue (25-35%) in its primary structure, no disulfide bridges are thought to exist in the native protein. Most species express two major isoforms (MT-1 and MT-2) which may be differentially expressed under different environmental conditions. Another salient feature about the primary structure is the absence of aromatic amino acids.

Substantial speculation exists with regard to the functional significance of metallothioneins. However, their involvement in copper and zinc metabolism is well documented. Induction of the gene occurs at the transcriptional level by many factors, including dietary zinc intake, heavy metal administration, glucocorticoid hormones and cytokines, which have been well described (Cousins, 1985, Hamer, 1986, and Dunn, et al., 1987). However, changes in messenger RNA stability and processing for subsequent translation are points where further regulation is possible. This may explain why high levels of metallothionein gene transcription are measured in tissues.
like the placenta, but low levels of the translated protein are observed (Huber and Cousins, 1988).

Transcriptional regulation of metallothionein gene expression by specific metal ions is thought to involve the binding of the metal to an ill-defined metal regulatory protein (MRP), which then binds to the metal regulatory element (MRE) in the promoter region of the gene (Hamer, 1986). The essential metal-specific transcription factors bind to the MRE region of the DNA to facilitate transcription. This metal regulatory element has been sequenced and its function confirmed (Carter et al., 1984). A nuclear protein with MRE-binding properties has been identified in yeast and partially purified from mouse cells (Imbert et al., 1989). Searle (1990) has identified a similar protein in nuclear extracts from rat liver, zinc-activated protein (ZAP), believed to be responsible for the zinc-responsiveness of the metallothionein genes.

Zinc deprivation during gestation leads to profound, and irreversible impairment of immunologic ontogeny that persists through three generations, in addition to the well-documented and profound developmental aberrations (Beach et al., 1983). Using a multi-generational murine model it was shown that gestational zinc deprivation alters normal zinc metabolism and the response of metallothionein to various stimuli throughout life (Vruwink et al., 1988). Some of these changes may be mediated by changes in metallothionein
gene expression in the fetal liver, although these authors did not investigate this possibility.

The developmental regulation of the metallothionein genes has been well investigated. Regardless of gestational zinc exposure, fetal liver metallothionein concentrations increase rapidly beginning on the eighteenth gestational day (Mercer and Grimes, 1986), peak at parturition and decline steadily reaching adult levels by weaning (Vruwink et al., 1988). The fetal rat liver metallothionein expression responded proportionally to graded levels of maternal zinc intake during the last 7d of gestation, as well as to interleukin-1 administration (Huber and Cousins, 1988). It is noteworthy that at this stage of gestation (G18-birth), the fetal liver is the primary hematopoietic organ of the rat, suggesting the possible influence of dietary zinc status on hematopoiesis.

Zinc and Cytokines

For many years the role of zinc in the immune function has been investigated with mixed results. An infectious insult causes dramatic and rapid physiologic changes, and alters the normal distribution of zinc in the body. Normally, the lung, heart, muscle and brain maintain relatively constant zinc concentrations, regardless of dietary zinc status or hormonal fluctuations. However, dramatic changes occur in skin, liver, bone marrow, thymus and blood zinc levels during infection or stress (Cousins
and Leinart, 1988, Huber and Cousins, 1988, Dunn and Cousins, 1989). Like iron, zinc is important to both micro-organism survival and immune functions (Weinberg, 1974). Both metals are rapidly removed from the plasma and taken up in large amounts by the liver (Klassing, 1984). Iron is stored in an unavailable form as ferritin or hemosiderin and is also taken up by macrophages and stored as lactoferrin (Goldblum et al., 1987). The cytokines induce the synthesis of metallothionein in the liver and as a result zinc is sequestered in the liver. This zinc-metallothionein may be a labile source of zinc available for intracellular exchange.

Upon initiation of an infection, interleukin-1 is released from immunoactive cells, like macrophages and monocytes. It induces a cascade of responses, including the production of other cytokines (e.g. IL-6), growth factors, antibodies, and the rapid proliferation of lymphocytes, and stem cell differentiation (Dinarello, 1988). Together these molecules orchestrate a broad spectrum of systemic responses commonly referred to as the "acute-phase response."

Increased synthesis and release of acute-phase proteins from the liver occur (e.g. ceruloplasmin, c-reactive protein, serum amylase A), accompanied by decreased albumin and transferrin synthesis. Large concentrations of copper and zinc have been shown to influence secretion of IL-1β, tumor necrosis factor and IL-6 from blood monocytes in vitro.
(Scuderi, 1990). For complete reviews regarding the biological effects of interleukin-1, the reader is referred to Kampschmidt, 1984, and Dinarello, 1988.

The increased amount of zinc appearing in the marrow may be utilized in the mechanisms of clonal expansion and cell differentiation as described earlier. It is also possible that zinc in marrow may be available to enzymes involved in cellular proliferation through ligand exchange from metallothionein. The flux of zinc to these organs may also act to stabilize membranes, alter membrane fluidity, influence receptor binding, or augment phagocytic cell motility in accord with previously discussed mechanisms.

Winchurch (1988) has provided evidence that zinc potentiates the effect of interleukin-1 in thymocytes in vitro and suggests that the zinc interaction occurs early in the proliferative response, possibly enhancing the cellular uptake of interleukin-1. It is possible that zinc may be internalized with the interleukin-1 receptor and released into the cytosol which could trigger the induction of metallothionein. The family of cell surface proteins described by Cunningham et al. (1990) also supports this finding.

Schroeder and Cousins (1990) demonstrated that interleukin-6 stimulates the expression of metallothionein only in the presence of dexamethasone in hepatocytes and its effect is potentiated by supplemental zinc in the culture
medium. These data suggest that interleukin-1 induces the production and release of interleukin-6, which is the cytokine to effect the zinc-related phase of the acute-phase response in the liver.

Certain hematological changes have been attributed to various cytokines. The role of interleukin-1 in hematopoiesis has been reviewed by Fibbe and Willemze (1991). For example, following a single injection of interleukin-1, increased numbers of circulating neutrophils and decreased numbers of lymphocytes were measured in the peripheral blood of rats (Ulich et al., 1987). This same group investigated the hematological effects of chronic interleukin-1 administration, concluding that this cytokine significantly induced myeloid hyperplasia and lymphopenia in the bone marrow (Ulich et al., 1989).

Interleukin-6 administration to rats, on the other hand, showed a significant increase in the synthesis of intermediate and late stage normoblasts between 12-24h, as well as a mild myeloid hyperplasia (Ulich et al., 1991). There has been some suggestion that these cytokines (and others) are involved in the regulation of hematological changes during inducible hematopoiesis. Interleukin-1 has been suggested to act as an erythroid suppressive factor. However, when recombinant interleukin-1α was tested, no effect on in vitro CFU-e colony formation was measured (Furmanski and Johnson, 1990). Although, these authors go
on to suggest that the erythrosuppressive effects attributed to macrophages may be mediated through the tumor necrosis factor released in response to IL-1 (Johnson et al., 1980).

**Zinc and Bone Marrow**

The bone marrow is one of the most highly organized organs of the body, accounting for up to 5% of total body weight (Gulati et al., 1988). Upon gross inspection, the bone marrow is a blood-like gelatinous substance which fills approximately 85% of bone cavities. Characterization of the marrow has been done primarily by hematologists and immunologists, based on its usefulness in clinical diagnosis and its primary function to generate the blood cells of the body's immune system. In consequence, little nutritional work beyond the importance of iron, vitamin B₁₂, and folate to erythropoiesis and cell replication has been done (Krause, 1988). Structurally the marrow has a highly innervated vascular compartment and an extravascular compartment where the hematopoietic cells adhere to a lattice structure made of endothelial and reticular cells, sometimes called hematopoietic cords.

Hematopoiesis in humans begins in the yolk sac around 3-4 weeks gestation, but declines by the middle of the third month, when the fetal liver takes over this function. The maturing bone marrow begins active hematopoiesis by the end of the fifth lunar month, increasing until maximal levels
are reached at birth and it becomes the primary hematopoietic organ (Gulati et al., 1988). Metallothionein production in the fetal liver follows a similar pattern, reaching maximal levels at birth, and declining soon thereafter to normal adult levels in liver (Panemagalore et al., 1983).

Daily red blood cell production by the bone marrow is estimated at 2.5 billion cells/d/kg body weight. Erythropoiesis from early precursor cells to reticulocytes takes 3-5 days in humans. Maturation begins with the mitotic division of a pronormoblast into two basophilic normoblasts. Three successive divisions lead to 16 polychromatophilic normoblasts; each time the cells become smaller and the nucleus becomes more dense. Finally, there is maturation without division to orthochromic normoblasts, and the nucleus is extruded and phagocytized by the surrounding macrophages. The new reticulocytes will remain in the marrow 1-2 days before entering the circulation.

Erythropoiesis is thought to be regulated by at least two independent mechanisms: stem cell-type regulation (e.g. IL-3) and pathway-specific regulation (e.g. erythropoietin) (Sassa et al., 1979). Two classes of progenitors, early (BFU-e) and late stage (CFU-e) erythroid progenitors, have been identified and characterized. BFU-e or early stage cells are the more primitive stem cell progenitors and are subject to stem cell-type regulation by factors like IL-3.
These cells are not normally in active cycle, as demonstrated by survival to tritiated thymidine. However, they do enter a proliferative stage following irradiation. CFU-e are late stage progenitors subject to pathway specific regulation and are normally in active cycle. Transformed cell lines and the clonal expansion assay of primary stem cells in semi-solid media containing different purified growth factors have been useful in elucidating these regulatory pathways (Metcalf, 1984).

The effects of metals, other than iron, on erythropoiesis have not been well studied. Knowing that cadmium toxicity induces iron deficiency anemia, Sakata and co-workers (1988) investigated whether it had a direct effect on erythropoietic parameters in vitro and/or in vivo. Using the methylcellulose assay, they determined that in vitro cadmium exposure depressed the growth of CFU-e (late) progenitors in a dose-dependent fashion, which could not be overcome by adding increasing amounts of erythropoietin to the cultures. However, the addition of metallothionein prevented the in vitro growth suppression. In these experiments, the cadmium treatment induced iron deficiency anemia in intact rats. Plasma erythropoietin concentrations, iron-binding capacities and CFU-e densities increased, while plasma iron concentration decreased. These effects could be prevented by simultaneous iron administration. These data suggest that the negative effect
of cadmium measured in vitro is caused by its cytotoxic effect on the cells. The in vivo changes are mediated by the induction of an iron deficiency state, rather than as a direct effect of the cadmium metal on erythroid cell development. The mechanism of this effect is unknown.

The regulation of erythropoiesis has also been studied in murine and human cell lines. The mouse Friend leukemia virus-transformed cell line contains a homogeneous population of erythroid progenitors and has been well studied (Sassa et al., 1979). The K562 line is a human erythroleukemic line, derived from a tumor, which perpetuates due to the presence of the Philadelphia chromosome in the genome of the cells, rather than through the insertion of a virus. Cells of this line are multipotential stem cells, and can be induced to differentiate into either granulocytes or erythrocytes (Lozzio et al., 1981). Erythroid differentiation can be induced within 48h by the addition of DMSO or hemin (among other compounds) and is marked by the appearance of specific cell surface antigens, spectrin synthesis and hemoglobinization (Sassa et al., 1979).

The individual effects of cytokines on clonal expansion of marrow progenitors have been facilitated by the advent of synthetic peptides. A recent study described growth suppression of CFU-e and BFU-e progenitors from K562 cells by the in vitro addition of tumor necrosis factor (TNF) in picomolar concentrations. Simultaneous incubation with
heroin alleviated this effect (Li et al., 1989). Receptors for tumor necrosis factor were present on the earliest progenitor cells, but they were not found on the more mature differentiating cells. TNF is also known to suppress the growth of HL-60 cells, a myelogenous leukemic human cell line, in similar concentrations. These data suggest that the more differentiated cells lose their ability to respond to this cytokine and perhaps other cytokines. It is possible, that certain cytokines only effect changes on the earliest progenitor cells, while others may effect changes in the more mature cells.

**Zinc and Radioprotection**

Ionizing radiation induces a number of symptoms that can be as mild as nausea, as moderate as a decrease in immune functions and as severe as death. Radiation damage to cells is primarily the work of free radicals, which can in turn produce harmful lipid peroxides or damage to DNA. Commonly recognized free-radical scavengers include superoxide dismutase, catalase, glutathione, glutathione peroxidase and vitamin E.

A role as a free-radical scavenger has also been postulated for metallothionein, based on its high thiol content. Many in vitro data are available to support a free-radical scavenger role for metallothionein, yet in vivo evidence has been less forthcoming. Zinc has been shown to confer a cytoprotective effect to red blood cell ghosts.
incubated with exogenous Fe(III) and xanthine compared to control ghosts not supplemented with zinc (Girotti et al., 1986), perhaps through an induction of metallothionein (Thomas et al., 1987). Although basal liver concentrations of glutathione are much greater (10 mM) than metallothionein (3 µM), metallothionein levels can be induced up to 4 mM by injection of metal salts (Thornalley and Vasek, 1985). These workers used spin trapping techniques to demonstrate the ability of Zn-MT to reduce hydroxyl radical formation, leading them to hypothesize a role for metallothionein in quenching oxidative stress. A dose-dependent protection against hydroxy-radical induced DNA damage by metallothionein has been described from in vitro studies, such that a 13 µM concentration of metallothionein proved equally protective against DNA damage as 10mM glutathione (Abel and Ruiter, 1989).

Using a primary culture hepatocyte model, a combination of 48 µM zinc, 1 µM dexamethasone phosphate and 10 ng/ml rhIL-6 was shown to protect cells from CCl₄ toxicity and was associated with high levels of metallothionein gene expression. Conversely, cells cultured without exogenous zinc in the medium were not protected from CCl₄ induced cell damage and death (Schroeder and Cousins, 1990). A free radical mechanism is thought to be responsible for the toxic properties of CCl₄. Bakka et al. (1982) demonstrated that cultured cells producing high levels of metallothionein were
resistant to ionizing radiation. However, other workers provide evidence that metallothionein is efficient in protecting Chinese hamster ovary cells against damage from alkylating agents, but not free radicals generated by ionizing radiation (Kaina et al., 1990).

Ionizing radiation and high oxygen tension over several days have been shown to induce the synthesis and accumulation of metallothionein in rats in vivo (Shiraishi et al., 1983). Substantial evidence suggests that the injection of mineral salts, which induce metallothionein, 1-2 days prior to irradiation confers protection against radiation-induced death in mice and rats (Floersheim and Floersheim, 1986, Matsubara et al., 1986, 1987, 1988). Ionizing radiation doses, as low as 2 Gy, induce metallothionein in an organ specific manner (Shiraishi et al., 1989, Koropatnick et al., 1989). The former group provided evidence of induction in the liver, kidney and thymus, while the latter group only recorded increased expression in the liver. Koropatnick et al. (1989) demonstrated that there was a dose-dependent increase in metallothionein in the liver with increasing radiation exposure; however, liver zinc concentrations did not increase. This would suggest that induction of metallothionein by radiation injury occurs by a factor other than zinc. It may be hypothesized that factor is a cytokine
released in response to the tissue injury or a tumor secretion, in the case of a malignancy.

Zinc salts are being investigated as radioprotective agents. Floersheim et al. (1988) provided evidence that pretreatment with 30 mg Zn-aspartate/kg body weight to mice infected with tumors led to a positive effect on recovery and survivability from ionizing radiation, without affording protection to tumor cells. They demonstrated that administration of zinc aspartate, within 30 minutes of irradiation, reduced the characteristic fall in the hematocrit and the numbers of circulating erythrocytes, white blood cells and thrombocytes. Many organic zinc salts were shown to confer radioprotection; however, zinc aspartate (10 mg/kg) was the only salt which did not also inhibit regression of tumor growth (Floersheim and Bieri, 1990). Since metallothionein induction in the liver requires more than 30 minutes, these authors discount metallothionein as the radioprotective agent. It is possible to speculate, however, that additional zinc is used to maintain the proper tertiary structure and stability of the existing metallothionein molecules, so that cellular repair processes may occur. Additionally, changes in the bone marrow may occur more rapidly, thus offering better recovery of the hematological parameters which are described by these authors.
Other studies in radioprotection focus on the protective role of the cytokine interleukin-1. Among the biological roles postulated for interleukin-1 in the inflammatory process, is the induced cycling of early and late stem cell progenitors in the bone marrow (Neta et al., 1987). Further work demonstrated better, although not complete, recovery of these cells when mice were pretreated with the cytokine before irradiation (Schwartz et al., 1988). The specificity of this effect has been confirmed by in vitro work comparing protection by IL-1, IL-2 and IL-3 (Gallicchio et al., 1989). Only interleukin-1 conferred radioprotection and its effect could be blocked by simultaneous incubation with an antibody to IL-1.

The mechanism of radioprotection by interleukin-1 may involve the induction of stem cell progenitors into proliferation and differentiation. However, the possible induction and role of acute phase proteins, like metallothionein, in progenitor cells has not been well investigated. Increased amounts of zinc are redistributed to the marrow following interleukin-1 administration to rats and increased levels of metallothionein mRNA have also been measured in marrow in response to interleukin-1 (Huber and Cousins, 1989 abstr). This could provide a mechanism for cells to acquire more zinc to serve a protective role.
CHAPTER 3
MATERIALS AND METHODS

Materials and Diets

Male Sprague-Dawley rats (Harlan Breeding Laboratory, Indianapolis, IN) were used for all in vivo experiments. Rats arrived weighing 75-100g and were allowed to acclimate for a 7d period, in which they were provided free access to water and the Teklad 22/5 Rodent Diet (W) (Madison, WI) containing 51 mg zinc/kg diet, as zinc oxide. Husbandry involved individual cages in a room with a 12 hour light/dark schedule, maintained at 22-25°C. Rats were killed by cardiac puncture under Metofane (Pitman-Moore, Washington Crossing, NJ) inhalant anesthesia at the times indicated. Care and treatment of experimental animals received prior institutional approval and followed NIH guidelines (NIH pub. #85-23).

For the dietary studies, rats were given free access to a pelleted purified diet formulation (Research Diets, New Brunswick, NJ) based on the AIN-76A diet (American Institute of Nutrition, 1977, 1980) containing 30 mg Zn/kg diet and double-distilled deionized water for a 7d acclimation period. Rats were randomly assigned to diets modified to contain 5, 30, or 180 mg Zn/kg diet, representing marginally
deficient, adequate and supplemental levels of dietary zinc. As described previously (Blalock et al., 1988), the purified diet contained 6 mg Cu/kg diet as cupric carbonate and the zinc content was adjusted with zinc carbonate. The zinc content of the diets was verified by dry ashing and atomic absorption analysis (data not shown).

Reagents and Materials

All solutions and tissue culture media were prepared with double-distilled, deionized water and sterilized by autoclave when necessary. The interleukin-1α (Lot 1/87; specific activity= $2.5 \times 10^9$ D10 Units/mg protein) was a gift from Hoffmann-LaRoche (Nutley, NJ). The interleukin-6 (Lot1/89; specific activity= $1 \times 10^6$ D10 Units/mg protein) was provided by Genetic Institute (Cambridge, MA). The osmotic pumps (Model Alza 2001) were purchased from Alza Corporation (Los Angeles, CA). [$\gamma^{32}$P]-ATP (6000 Ci/mmol), [$\alpha^{32}$P]-CTP (6000 Ci/mmol); $^{65}$Zn (18 mCi/mg Zn) and GeneScreen nylon filter membranes were purchased from Du Pont New England Nuclear Products, (Boston, MA). The carrier-free $^{109}$Cd was purchased from Isotope Products Laboratories, (Burbank, CA). RPMI 1640 tissue culture media and heat-inactivated fetal calf serum, T4 polynucleotide kinase, and Random primer extension labeling kit were purchased from Bethesda Research Laboratories/GIBCO, (Gaithersburg, MD). All other chemicals used were at least reagent grade and
were obtained from Fisher Scientific Co., (Pittsburgh, PA) or Sigma Chemical Co., (St. Louis, MO). Statistical software for the personal computer was purchased from SAS Institute (Cary, NC) and GraphPAD version 1.10a by InStat (San Diego, CA).

Cell Line

K562 (ATCC CCL 243) cell line was established from the pleural effusion of a 53 year-old woman with chronic myelogenous leukemia in terminal blast crisis by Lozzio and Lozzio (1975). This is an erythroleukemic line of multipotential cells that can spontaneously differentiate into recognizable mature erythroid, granulocytic and monocytic cells. These cells can be specifically and reversibly induced to differentiate along the erythroid cell lineage (Dean et al., 1981). However, the cells will not reach functional maturity or extrude the cell nucleus.

General Methods

Tissue Collection

Bones were removed, cleaned and dropped directly into liquid nitrogen and stored at -70°C. Livers were excised and frozen at -70°C. The frozen bones were split with a razor blade, the frozen marrow removed and homogenized in 10 mM Tris buffer with a polytron grinder for the assay of metallothionein. Frozen liver tissue was weighed and prepared, as was the marrow. Fresh marrow and liver were
removed and dropped into 4.5 M guanidinium isothiocyanate for immediate extraction of total RNA (Chomczynski and Sacchi, 1987). Bone marrow was removed from the femur and tibia of both legs of the rat to constitute one sample for RNA extraction or protein determination. Because of the limited sample size available from the bone marrow, MT mRNA, metallothionein protein, and tissue zinc determinations could not be performed on the same animal. Bone marrow from 3 rats was pooled, weighed and digested with 1:3 nitric to sulfuric acid in a hot bead bath to assess zinc levels by atomic absorption spectrometry.

Cell Culture

K562 cells were maintained in continuous suspension culture at 2.5 x 10^5 cells/ml RPMI 1640 medium, supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 2.0 g sodium bicarbonate/L, penicillin G (0.1 U/L) and gentamycin (65 µg/L). Cell doubling time was 24 hours, with media changes every two-three days. Periodically, K562 cells (10^6 cells/ml) were stored at -70°C in 10% DMSO, 20% FCS and antibiotic-free media.

Experiments under defined culture conditions used cells plated in 100 mm³ dishes at a concentration of 2.5 x 10^5 cells/ml in 20.0 ml of medium to insure log phase growth. Incubation times were defined by the specific experimental protocol. Harvesting cells involved washing the cells three
times with 20 ml of 1X PBS (pH 7.4) buffer and pelleting by centrifugation at 500 x g for 10 min. For $^{109}$Cd-hemoglobin saturation assays, $5 \times 10^6$ cells were resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.4) buffer. Aliquots (20 µl) were taken to measure cell protein. Duplicate plates were resuspended following washing in 0.2 N NaOH (8.0 g/L) and 0.2% SDS and allowed to incubate overnight at room temperature before measuring cellular zinc concentration. RNA extraction required approximately 12 to 25 x $10^6$ cells, which were lysed in 4.5 M guanidinium isothiocyanate, and extracted as described above. Cell counts and viability determinations were done routinely using the exclusion of the vital dye, trypan blue.

Statistical Analysis

Tissue and cell metallothionein protein and gene expression data were analyzed by one-way analysis of variance and Bonferoni's Corrected p value test for all studies, except the dietary zinc and cytokine interaction study. Two-way analysis of variance was conducted for the dietary zinc and cytokine interaction study, followed by Duncan's Multiple Range tests using the SAS statistical computer package.
Biochemical Analyses

Atomic Absorption Spectrophotometry

Zinc concentrations in serum and media were measured by air/acetylene flame atomic absorption spectrophotometry (AAS). Serum samples were diluted 1:5 with deionized, distilled water (ddH₂O) and concentrations measured using standards of zinc in 1% HCl. Media and cell samples were diluted appropriately to record zinc measurements in the standard range. Sample concentrations were calculated using linear regression from the standard concentration line.

Protein Quantitation

Protein measurements in all experiments were done by the folin phenol reagent method of Lowry et al. (1951). Appropriate dilutions of a sample were made and compared to a bovine serum albumin (BSA) standard curve by linear regression analysis of absorbance at 500nm after 30 min. Protein determinations on pooled fractions of marrow separated on Percoll gradients were measured using the Bio-Rad Bradford protein assay, since concentrations were below 25 mg/ml.

Metallothionein Levels

Metallothionein protein levels were measured using the ¹⁰⁰Cd-hemoglobin saturation assay (Eaton and Toal, 1982). Briefly, a known specific activity of the ¹⁰⁰Cd isotope is added to a heat-denatured cytosol homogenate (MT is heat-
stable), and allowed to incubate 10 min. Metallothionein has a stronger affinity for cadmium, such that the zinc is displaced. Hemoglobin, having a strong affinity for cadmium, is used to precipitate the remaining free $^{109}$Cd through heat denaturation. Using a gamma ray spectrometer to monitor the radioactivity in a measured aliquot of sample, one can calculate the $\mu$g MT/g tissue (or per mg protein) based on the specific activity of a given sample.

**Liquid Chromatography**

Molecular weight separation of tissue and cell homogenate cytosols was accomplished using a 16 x 2 inch column of Sephadex G-75 gel. The column was determined to have a void volume ($V_0$) of 22 ml and a total volume ($V_t$) of 62 ml, as determined using Blue Dextran 2000 and $B_{12}$ standards. Flow was driven by a Gilson peristaltic pump at 0.5 ml/min. Prior to application of experimental cytosols, a control cytosol was run through the column. Metallothionein characteristically elutes at $1.8V_0$ or approximately 40 ml for this column apparatus.

Briefly, all cytosols were prepared in 1 mM Tris-HCl (pH 8.0) and approximately 1.0 ml was loaded to the column and eighty 1.0 ml fractions were collected. Lowry protein determinations on cell cytosols were made to standardize experimental cytosols for comparison to one another. Each fraction was analyzed for zinc concentration using atomic
absorption spectroscopy and protein concentration by measuring absorbance at 280nm.

RNA Extraction & Hybridization

The RNA extraction procedure of Chomczynski and Sacchi (1987) and hybridization techniques of Berent et al. (1985) were used. These procedures have been described (Huber, thesis, 1988).

Total RNA was obtained by phenol/chloroform/isoamyl alcohol extraction following homogenization in a 4.5 M guanidinium isothiocyanate solution to which 4.5 M sodium acetate was added. The mixture was centrifuged 10,000 x g for 20 min at 4°C, the aqueous phase was transferred to a fresh tube, and the total RNA was precipitated with isopropyl alcohol for 1h at -20°C. The pellet was washed with 75% ethanol, before being dissolved in sterile DEPC-treated ddH₂O, and stored at -20°C. Absorbance spectra and the 260nm/280nm ratios were measured, and the sample re-extracted when the ratio did not approximate the desired 2.0.

Total RNA was electrophoresed through 1.5% agarose in 0.1 M sodium phosphate (pH 7.4) at constant voltage (45V until it passed the wells, then 90V for 2h). Before transfer of the RNA to a solid matrix, the integrity of the 18S and 28S ribosomal RNA bands was determined using a UV-light box to visualize ethidium bromide staining in the gel.
The RNA was transferred to nylon membranes (GeneScreen or Nytran) using a vacuum blotter at 5 psi in 10X SSC buffer (pH 7.0) for 1.5h. Without allowing the nylon to dry, the RNA was cross-linked to the membrane using a Hoeffer UV-cross-linker for 3.4 min. At this time the blots were either hybridized to radiolabeled probes or stored at 4°C for further analysis. Following a stringent washing procedure in 10X SSC and 1% SDS (30 min at 90-100°C), membranes which were not allowed to dry could be used for multiple hybridizations.

The DNA probes used for hybridizations were 60 base synthetic oligonucleotides corresponding to the 5' region of the metallothionein gene which has been cloned and sequenced from humans, mice, and rats (Durnam et al., 1980). [γ-32P]-ATP and the 60-mer probe were incubated with T4 kinase and the labeled probe purified by gel filtration. Routinely, the specific activity of the probe was 9.8 x 10^8 cpm/µg. For analysis of metallothionein gene expression in K562 cells, cDNA probes for metallothionein and β-actin were labeled using DNA polymerase and the random primer extension method. Probes were added to hybridization solution for 16-24h. The filters were washed to remove any non-specifically bound probe and placed at -70°C with Kodak XAR-2 film and intensifier screens for visualization.
CHAPTER 4
ZINC METABOLISM IN THE BONE MARROW

Before investigations regarding dietary zinc/cytokine interactions or changes in particular cell populations within the marrow could be conducted, basic characteristics of zinc and metallothionein in the bone marrow had to be defined. To our knowledge, these are the first direct investigations regarding dietary zinc metabolism and metallothionein in the intact rat bone marrow. However, measurements of metallothionein and zinc in the red blood cells have been made in response to cadmium, endotoxin and iron deficiency, respectively (Tanaka et al., 1986; Morrison et al., 1988; Robertson et al., 1989) using a variety of analytical methods.

The presence and zinc responsiveness of the metallothionein genes in the bone marrow was investigated using two protocols: acute administration of zinc and chronic zinc exposure by the implantation of mini-osmotic pumps. The specificity of the response was investigated using dexamethasone phosphate administration, in both cases. Changes in zinc and metallothionein levels in response to varying nutritional zinc status using rats maintained on marginally deficient, adequate and supplemental zinc intakes were also investigated. Responses to treatments were
measured using plasma and tissue zinc analysis by air/acetylene atomic absorption spectroscopy, metallothionein expression by indirect assay, chromatography and Northern analysis of metallothionein mRNA.

Acute and Chronic Zinc Administration

Specific Experimental Protocols

Rats were fed a standard commercial diet, as described in Chapter 3, for 7d prior to assignment to the experimental protocol. For acute zinc administration experiments, either 25 µmol zinc (as zinc sulfate), 2 mg dexamethasone (sodium phosphate)/kg body weight or an equivalent volume saline was injected (i.p.). Rats were killed either 6h (n=3) after injection to collect tissues for analysis of MT mRNA, or 16h later (n=3) to measure metallothionein protein levels. Serum zinc concentrations were also measured.

Another group of rats were used for a 7d experiment involving implantation (i.p.) of the Alza Mini-Osmotic pump 2001 for continuous infusion of zinc sulfate (n=6) or dexamethasone phosphate (n=5). A sham implantation operation was performed on controls (n=6). Pumps releasing saline were not used, however, because of cost considerations.

Prior to initiating implantation surgery, all pumps were filled with either the zinc sulfate or dexamethasone solution. These pumps have a continuous flow rate of 1 µl/hr, beginning approximately 4h after implantation. The
zinc dose was designed to deliver approximately 24 µmol zinc/d (0.37 µg zinc/d) at 1 µmol/h. The dexamethasone concentration was 3.2 mg/ml and delivered approximately 77 µg/d. The pumps can be weighed before and after filling to insure volumes and contain roughly 0.2 ml solution. These doses were selected in order to approximate the dose levels used to induce metallothionein in the liver by a single dose of zinc (Richards and Cousins, 1975) or dexamethasone (Etzel et al., 1979).

Implantation was performed under sodium pentobarbital anesthesia. Briefly, a 2 cm incision through the skin and the peritoneal cavity was made and the pump was inserted flow moderator first, towards the left ventral side of the cavity. A continuous line suture with sterile 3.0 silk was used to close the peritoneal cavity and wound clips closed the skin. All rats were closely observed during recovery, according to animal care guidelines. After full recovery, animals were returned to the animal facility, individually housed and given free access to water and diet. Following the 7d infusion period, rats were killed by cardiac puncture and blood and tissues were collected. Serum zinc concentrations and metallothionein levels in liver and bone marrow were measured.

Results

The acute zinc treatment was able to increase marrow metallothionein levels to 93 ng MT/mg protein compared to 58
ng MT/mg protein for saline injected controls by 16h. Data represent the pooled measurements from three rats/treatment. Dexamethasone phosphate treatment decreased marrow metallothionein to 42 ng MT/mg protein. Within 6h of treatment, serum zinc concentrations were significantly different between groups (mean ± SD) 1.5 ± 0.1, 2.5 ± 0.2 and 1.4 ± 0.2 µg Zn/ml in control, zinc and dexamethasone groups, respectively. By 16h, serum zinc concentrations had returned to control levels: 1.5 ± 0.1, 1.4 ± 0.2 and 1.7 ± 0.2 µg Zn/ml. Liver metallothionein was increased by both zinc treatment (74 ± 19 µg MT/g) and dexamethasone (35 ± 4) compared to the control group (18 ± 1), as expected.

A similar protocol was conducted for chronic zinc and dexamethasone exposure, as described above. In these experiments, statistically significant differences between the groups were not found (Table 4-1). However, chronic dexamethasone phosphate release from the mini-osmotic pump had significantly detrimental effects on the rats, as estimated regarding weight loss and overall well-being. Metallothionein concentrations in the dexamethasone treated marrow were 23 ± 8 ng MT/mg protein, compared to 53 ± 6 for the control group, and 51 ± 14 for the zinc treated group. Serum zinc concentrations in the zinc treated group (1.7 ± 0.5 µg Zn/ml) were significantly higher than the control (1.2 ± 0.3) and dexamethasone treated groups (1.1 ± 0.1).
Rats were randomly assigned to treatment (TRT) groups, following a 7d acclimation period to the new surroundings. Control rats were sham-operated, Zinc and Dex rats were implanted with mini-osmotic pumps for 7d, as described in Specific Experimental Protocols. Serum zinc, liver and marrow metallothionein were measured as described in Chapter 3.

Table 4-1
Serum zinc, liver and marrow metallothionein values following 7d chronic zinc administration via mini-osmotic pump

<table>
<thead>
<tr>
<th>Rat #</th>
<th>TRT Group</th>
<th>Serum Zn µg Zn/ml</th>
<th>Liver MT ng MT/mg protein</th>
<th>Marrow MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1.4</td>
<td>724</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>1.2</td>
<td>701</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>1.6</td>
<td>801</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>0.9</td>
<td>1038</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>1.0</td>
<td>983</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>0.8</td>
<td>732</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>Dex</td>
<td>0.9</td>
<td>2249</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>Dex</td>
<td>1.2</td>
<td>3103</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Dex</td>
<td>1.3</td>
<td>1789</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>Dex</td>
<td>1.2</td>
<td>1262</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>Dex</td>
<td>1.2</td>
<td>3172</td>
<td>14</td>
</tr>
<tr>
<td>12</td>
<td>Zinc</td>
<td>2.1</td>
<td>2614</td>
<td>60</td>
</tr>
<tr>
<td>13</td>
<td>Zinc</td>
<td>1.5</td>
<td>1197</td>
<td>43</td>
</tr>
<tr>
<td>14</td>
<td>Zinc</td>
<td>2.2</td>
<td>4045</td>
<td>61</td>
</tr>
<tr>
<td>15</td>
<td>Zinc</td>
<td>1.9</td>
<td>1492</td>
<td>47</td>
</tr>
<tr>
<td>16</td>
<td>Zinc</td>
<td>1.9</td>
<td>7704</td>
<td>66</td>
</tr>
<tr>
<td>17</td>
<td>Zinc</td>
<td>0.9</td>
<td>896</td>
<td>30</td>
</tr>
</tbody>
</table>
Specific Experimental Protocols

For the dietary zinc studies, rats (n=5) were acclimated to the AIN-76A diet (American Institute of Nutrition, 1977, 1980) in pelleted form for 7d, and assigned to diets, adjusted with zinc carbonate to contain 5, 30 or 180 mg zinc/kg diet, for 14d (Research Diets, New Brunswick, NJ). Rats were sacrificed on d15 of the dietary regimen for analysis of serum zinc, metallothionein and metallothionein mRNA, as described in Chapter 3. Another group of rats (n=3) maintained on the 30 mg Zn/kg diet for 14d were sacrificed, and the marrow was pooled, a cytosol prepared and chromatographically separated on Sephadex G-75.

Results

Chromatography of the cytosol from bone marrow of rats fed the purified zinc adequate diet further substantiates the presence of appreciable amounts of metallothionein in the bone marrow (Figure 4-1). The metallothionein peak indicated by increased zinc is located at 1.8V₀ and coincides with almost no absorbance at 280nm, due to the absence of aromatic residues in the primary sequence of the protein. These chromatographic characteristics of metallothionein have been well established in this lab and others over the past thirty years.
Figure 4-1. Chromatographic separation of bone marrow cytosol on G-75 Sephadex. Bone marrow was from 3 rats was pooled, homogenized in 1 mM Tris-HCl (pH 8.0), loaded to a 16 x 2 in column of G-75 Sephadex and eluted with 1 mM Tris-HCl (0.2% sodium azide) at 0.5 ml/min at 4°C.
Absorbance at 280nm vs. Elution Volume (mL)

- Absorbance
- ZINC

Elution Volume (mL)

MT
Tissue zinc concentration in rats fed marginal, adequate, and supplemental dietary zinc intakes for 14d was measured by atomic absorption spectroscopy of tissue acid digests (Table 4-2). Bone marrow zinc concentrations were significantly decreased in rats fed the marginally zinc deficient diet for 14d. The zinc levels were not significantly increased in rats fed the supplemental zinc diet. These data suggest that the amount of zinc contained within the marrow compartment is sensitive to zinc depletion. Tissue zinc concentrations in the liver and kidney did not change over the dietary feeding period. The efficacy of the diets is shown by the serum zinc values (0.8 ± 0.1, 1.4 ± 0.2, and 1.7 ± 0.5) for the marginal, adequate, and supplemental zinc groups, respectively. Although rats on the 5 mg Zn/kg diet tended to gain less weight than their counterparts, weight gain was not significantly different among dietary groups (Table 4-2).

Tissue metallothionein levels reflected the zinc concentrations measured in the bone marrow, liver and kidney over the 14d feeding period, shown in Figure 4-2. Rats fed a marginally zinc deficient diet had significantly less marrow metallothionein (90 ± 17 ng MT/mg protein), compared to rats fed adequate and supplemental zinc intakes (108 ± 17 and 129 ± 15, respectively). However, as was seen in the
Table 4-2

Tissue zinc concentrations, serum zinc values, and weight gain of rats fed marginal, adequate and supplemental levels of dietary zinc for 14d.

<table>
<thead>
<tr>
<th>Dietary Zinc Content (mg Zn/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Marrow Zn</td>
</tr>
<tr>
<td>Liver Zn</td>
</tr>
<tr>
<td>Kidney Zn</td>
</tr>
<tr>
<td>Wt. Gain</td>
</tr>
<tr>
<td>Serum Zn</td>
</tr>
</tbody>
</table>

Tissue zinc concentrations were measured by air/acetylene flame atomic absorption spectrometry, following tissue digestion in 1:3 nitric:sulfuric acid in a hot bead bath for 24h. Serum zinc was measured by air/acetylene flame atomic absorption spectrometry, after 1:4 dilution with ddH<sub>2</sub>O. Data represent the means ± standard deviations (n=3). One-way ANOVA was conducted using GraphPAD by InStat, and groups were compared using Bonferoni's corrected p value. Means within rows having different superscripts are significantly different p<0.05.
Figure 4-2. Tissue metallothionein levels. Metallothionein was measured, as described in Chapter 3, in marrow (ng MT/ng protein), liver (ng MT x 10^{-1}/mg protein) and kidney (ng MT x 10^{-1}/mg protein) of rats fed marginally deficient, adequate and supplemental zinc for 14d. Data represent means of 5 determinations, SEM = 2 for the marrow, SEM = 5 for the liver and SEM = 17 for the kidney. * indicates a statistical difference p<0.05.
rats chronically exposed to high levels of zinc, both tissue zinc and metallothionein concentrations in the bone marrow tended to plateau with increased dietary zinc intake. Although the metallothionein levels measured in this dietary study exceeded the levels measured in the previous studies, metallothionein concentrations within a protocol are comparable to each other. Kidney metallothionein increased with increasing zinc in the diet (19 ± 4, 28 ± 9, and 41 ± 9 µg/g tissue), which follows previously published results (Blalock, et al., 1988, Huber and Cousins, 1988).

Metallothionein mRNA levels were measured in the liver, kidney and the bone marrow. However, obtaining undegraded messenger RNA from the bone marrow proved to be quite challenging. The Northern blot, shown in Figure 4-3, demonstrates the presence of metallothionein mRNA in the bone marrow. However, any conclusions regarding its quantification would be presumptuous and based upon speculation, owing to the wide variability encountered.

Discussion

These studies have demonstrated the presence of metallothionein in the bone marrow. Indirect evidence is also presented, which suggests that the metallothionein genes respond to changing zinc levels by inducing the synthesis of this protein in the bone marrow. Unlike the liver, dexamethasone has the reverse effect, appearing to down-regulate metallothionein in the bone marrow.
Figure 4-3. Northern blot analysis of metallothionein mRNA. Rats were fed marginally deficient, adequate or supplemental zinc intakes for 14d. Total RNA was extracted from marrow, liver and kidney; 10 µg RNA/lane was fractionated through 1.5% agarose. Hybridization was to $^{32}$P end-labeled oligonucleotides specific for metallothionein and β-actin.
Although acute zinc treatment by injection specifically increased the amount of marrow metallothionein, chronic exposure to very high levels of zinc by the mini-osmotic pump did not increase metallothionein levels in the marrow. One explanation for this finding is that the pumps releasing the zinc malfunctioned. Upon post-study inspection, the dexamethasone pumps were clean and empty. The pumps containing the zinc sulfate had become encapsulated with lymphatic tissue and salt precipitation was evident in the peritoneal cavity. Nevertheless, the presence of the zinc was able to increase serum zinc and liver metallothionein values, suggesting that this was not a technical impediment. Another explanation is the variability associated with measurements of marrow metallothionein.

When circulating zinc levels were increased by acute zinc administration, the marrow had the capacity to up-regulate the expression of metallothionein almost two-fold. The results from experiments with chronic exposure to high levels of zinc indicate that perhaps an adaptive mechanism had occurred that limited very high levels of zinc and/or metallothionein from accumulating in the bone marrow during the 7d zinc exposure. The hormonal milieu of the 7d dexamethasone treated rats was considerably different than other groups and metallothionein levels in the marrow were much lower, even given the high variability in measurements.
In conclusion, these results suggest that amount of zinc accumulating in the bone marrow is highly regulated, but susceptible to depletion during restricted zinc intake. However, the variety of cell types present in the bone marrow complicate the interpretation of these data. It is possible that a particular cell type responds to increased zinc by induction of metallothionein, while another cell population fails to respond. Depending on the relative size of each cell population, this would significantly affect the measurement of metallothionein in the whole tissue.
CHAPTER 5
STRESS EFFECTS ON MARROW ZINC METABOLISM

Once the characteristic response of zinc metabolism and metallothionein in the bone marrow was established by the initial investigations described in Chapter 4, it was of interest to determine the responsiveness of the rat hematopoietic system to various stress inducers. We chose to investigate the response of zinc metabolism and metallothionein expression in the bone marrow using two models: in vivo administration of two cytokines released early on in the host-defense response (interleukin-1 and interleukin-6) and whole-body irradiation.

The hematologic effects of these cytokines have been documented in the literature, and have been discussed under the heading Zinc and Cytokines in Chapter 2. Briefly, interleukin-1 has been implicated as a mediator responsible for stimulating clonal expansion of the neutrophilic lineage, and increased numbers of late stage normoblasts are found in the bone marrow of interleukin-6 treated rats. Whole-body irradiation of 1.0 Gy (100 rad) is known to damage actively cycling cells in the marrow, decreasing numbers of CFU-e and increasing numbers of BFU-e progenitors. These hematological shifts may be important in the interpretation of the following studies.
Specific Experimental Protocols

The interaction between dietary zinc status and interleukin-1 and interleukin-6 was investigated using a 3 x 3 factorial design and measuring metallothionein gene expression in the bone marrow and the liver. Male weanling (75-100g) Sprague-Dawley rats were given free access to either 5, 30, or 180 mg Zn/kg diet and double-distilled deionized water for 14d. On 15d, rats were injected (i.p.) with 5 µg rhIL-1α, 5 µg rhIL-6 or an equal volume of 0.9% saline at t=0h. Rats were killed 6h later for RNA extraction and 16h later to determine metallothionein levels in the liver and bone marrow. Serum zinc concentrations were measured at both time points. All data represent the means ± SD of determinations from five rats, except where otherwise indicated.

Results

When metallothionein concentrations in the liver, kidney and bone marrow were analyzed by two-way ANOVA, a significant interaction between dietary zinc intake and cytokine treatment was only detected in the liver (p<0.0267; SEM = 13.3). The simple effects of diet and cytokine treatment are summarized in Table 5-1. Metallothionein levels increased significantly (p<0.0407; SEM = 281) with dietary zinc intake in the bone marrow, and in the kidney (p<0.001; SEM = 23.3), but failed to increase
Table 5-1

Simple effects of dietary zinc intake and cytokine treatment on metallothionein tissue levels in kidney, liver, and bone marrow at 16h.

<table>
<thead>
<tr>
<th>Diet Effect</th>
<th>Kidney</th>
<th>Liver</th>
<th>Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg Zn/kg diet</td>
<td>19 ± 3 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 5 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>380 ± 35 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 mg Zn/kg diet</td>
<td>46 ± 5 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 ± 15 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>589 ± 62 &lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>180 mg Zn/kg diet</td>
<td>85 ± 12 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 ± 31 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>874 ± 77 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine Effect</th>
<th>Kidney</th>
<th>Liver</th>
<th>Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48 ± 29 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>19 ± 10 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>553 ± 224 &lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>rhIL-1α</td>
<td>57 ± 40 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>52 ± 38 &lt;sup&gt;C&lt;/sup&gt;</td>
<td>667 ± 264 &lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>rhIL-6</td>
<td>45 ± 31 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>36 ± 19 &lt;sup&gt;B&lt;/sup&gt;</td>
<td>624 ± 256 &lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Metallothionein protein was measured 16h later by the 109Cd-hemoglobin saturation assay.

Simple effects were analyzed using the SAS statistical analysis program. Data are expressed as means ± standard deviations across either diet or cytokine treatment (n=9). Means within columns having different superscripts are significantly different (p<0.05). A significant interaction (p<0.03) between diet and cytokine treatment was measured in the liver only.
significantly in response to either cytokine. The simple effects in the liver are striking, but not valid in the light of a significant interaction between dietary zinc intake and cytokine treatment.

Metallothionein protein concentrations in the bone marrow increased directly with the amount of dietary zinc intake, as shown in Figure 5-1. This three-dimensional graph provides for the visualization of any interaction between dietary zinc and the cytokine treatments. Marrow metallothionein levels were 1.5- and 2.3-fold higher in rats fed 30 or 180 mg Zn/kg diet, respectively. Although a significant induction by cytokines was not measured, there was a tendency for marrow metallothionein levels in cytokine-treated rats to be higher than controls. However, in each case, the level of zinc in the diet appeared to have primary control over metallothionein levels. Interleukin-1α induced the largest amounts of metallothionein at each zinc intake level. These data suggest that a certain population(s) of cells within the bone marrow are responding to dietary zinc intake level. Furthermore, another, perhaps smaller population of cells may be responding to the cytokines used in these studies.

Metallothionein gene expression in the bone marrow was quite low compared to expression in some other tissues, and no significant trends were apparent. The Northern blot shown
Figure 5-1. Bone marrow metallothionein levels. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Metallothionein protein was measured 16h later by the $^{109}$Cd-hemoglobin saturation assay. Data represent the means of 3 determinations (SEM = 281).
in Figure 5-2 verifies the presence of active metallothionein genes in the bone marrow. However, the variability of metallothionein mRNA is quite high, as previously discussed, and does not correlate directly with measurement of marrow metallothionein protein levels.

The interaction between dietary zinc and cytokine treatment on metallothionein accumulation in the liver is illustrated in Figure 5.3. Dietary zinc intake significantly (p<0.0001) affected liver metallothionein levels 16h after cytokine treatment. For example, metallothionein levels were 2.5-fold higher in zinc supplemented rats than in adequate or restricted rats. Cytokine treatment also had a significant effect on liver metallothionein levels (p<0.0003). Interleukin-6 nearly doubled metallothionein levels compared to saline treated control rats, regardless of dietary zinc intake, by 16h after the injection. Metallothionein levels 6h following rhIL-6 were slightly higher (16 ± 10 µg MT/g tissue, 32 ± 8, and 66 ± 26 in marginally zinc deficient, adequate and zinc supplemented rats, respectively), suggesting that the metallothionein response to interleukin-6 had peaked by 16h after treatment. In contrast, interleukin-1α increased metallothionein levels at 16h by 1.5-fold in zinc deficient rats, and 3-fold in zinc adequate and supplemented rats. Comparatively, metallothionein levels 6h following interleukin-1 treatment in these rats were comparable.
Figure 5-2. Northern blot analysis of marrow MT mRNA. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Total RNA was extracted, 10 µg/lane was loaded and fractionated through 1.5% agarose. Hybridization was to $^{32}$P end-labeled oligonucleotides specific for metallothionein and β-actin.
Figure 5-3  Liver metallothionein levels. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Metallothionein protein was measured 16h later by the 109Cd-hemoglobin saturation assay. Data represent the means of 3 determinations (SEM = 13).
These data suggest that the metallothionein response to interleukin-1 lagged behind the interleukin-6 response, as might be anticipated.

The interaction between dietary zinc intake and cytokine treatment was also manifested in metallothionein mRNA levels in the liver. The Northern blot shown in Figure 5.4 shows that interleukin-1 was able to significantly induce metallothionein gene expression, regardless of dietary zinc intake. However, the induction of metallothionein mRNA by interleukin-6 appeared to be markedly linked to the level of zinc in the diet, as shown in Figure 5-4.

The simple effects of dietary zinc intake and cytokine treatment in the liver indicate a trend for metallothionein mRNA to increase in response to increasing dietary zinc intakes. The induction in metallothionein mRNA accumulation in the liver by the cytokines was very significant (p<0.001). Again, metallothionein gene expression in the bone marrow was too low to draw significant conclusions regarding response trends.

When differential expression of the metallothionein gene isoforms, MT-1 and MT-2 were measured, the marrow response to dietary zinc was clear (Figure 5-5). In addition, this Northern blot provides evidence that MT-1 is the primary metallothionein isoform expressed in the intact marrow. Both isoforms of the gene in the liver respond to
Figure 5-4. Northern blot analysis of liver MT mRNA. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Total RNA was extracted, 10 µg/lane was loaded and fractionated through 1.5% agarose. Hybridization was to 32P end-labeled oligonucleotides specific for metallothionein and β-actin.
Northern Blot Hybridization of MTmRNA
Marrow from Cytokine* Treated Rats Fed
Marginally Deficient, Adequate or Supplemental
Dietary Zinc for 14d

*5 µg/rat

Figure 5-5. Differential expression of MT-1 and MT-2 mRNA in the marrow. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Total RNA was extracted, 10 µg/lane was loaded and fractionated through 1.5% agarose. Hybridization was to 32P end-labeled oligonucleotides specific for metallothionein-1 and metallothionein-2.
dietary zinc intake, as shown by the autoradiograph in Figure 5.6. This is consistent to findings by Cousins and Lee-Ambrose (1992).

**Whole Body Irradiation Studies**

**Specific Experimental Protocols**

Rats acclimated to the surroundings and the commercial diet for 7d received 0, 0.1, 0.25, 0.5, 1.0 or 5.0 Gy of ionizing radiation administered in a single timed dose (6.5s-326s) by the Gammacell 40 $^{137}$Cs source located in the Health Science Center of the University of Florida. The Gammacell 40 was calibrated to deliver 7550 Rad/h (75.5 Gy/h) on 7/17/79. Using the decay correction table and the half-life of the $^{137}$Cs isotope (30y), the dose level at the time of these studies was 1.53 Rad/s (0.015 Gy/s). Rats were killed by cardiac puncture 4h following radiation exposure. Tissues were collected and stored at -70°C until metallothionein analysis. Plasma zinc, hematocrits and bone marrow SOD activity were also measured. Data represent means of three determinations at each radiation exposure.

A second study investigated the temporal response of metallothionein to the radiation exposure. Acclimated rats were irradiated at a constant level (0.25 Gy) and killed at each time point 0h, 2h, 4h, 8h and 24h later. Tissues were collected and stored at -70°C until analysis for metallothionein and superoxide dismutase activity. Plasma
Northern Blot Hybridization of MTmRNA
Liver from Cytokine* Treated Rats Fed
Marginally Deficient, Adequate or Supplemental
Dietary Zinc for 14d

*5 µg/rat

Figure 5-6. Differential expression of MT-1 and MT-2 mRNA in the liver. Rats were administered 5 µg rhIL-1α, rhIL-6 or 0.25 ml 0.9% saline at t=0h on d15, after being fed 5, 30 or 180 mg Zn/kg diet for 2 wk. Total RNA was extracted, 10 µg/lane was loaded and fractionated through 1.5% agarose. Hybridization was to $^{32}$P end-labeled oligonucleotides specific for metallothionein-1 and metallothionein-2.
zinc and hematocrit values were measured. Data represent the mean of three determinations at each time point. These experimental parameters were selected based on the report by Yamaoka et al. (1991) which measured increased superoxide dismutase activity in the bone marrow of rats subjected to low dose irradiation.

Results

Metallothionein concentrations in liver increased as ionizing radiation exposure increased, as would have been expected from the literature. Levels of metallothionein in the bone marrow, on the other hand, tended to remain fairly constant. Although there may be a slight tendency for metallothionein concentrations in this tissue to decrease at radiation exposures above 0.5 Gy, as shown by Figure 5-7.

Table 5-2 shows almost a two-fold increase in Cu,Zn superoxide dismutase activity after 0.5 Gy radiation exposure. Superoxide dismutase activity falls off dramatically at radiation exposures in excess of 0.5 Gy. This is in agreement with the findings of Yamaoka et al. (1991) both for the magnitude of increase and response to ionizing radiation dosage. Changes in blood parameters were not expected, and were not measured except four hours after radiation exposure (Table 5-2).

Metallothionein synthesis in response to a fixed level of ionizing radiation was also investigated (0.25 Gy), and
Figure 5-7. Metallothionein levels following increasing radiation exposure. Rats were exposed to 0, 0.1, 0.25, 0.5, 1.0 and 5.0 Gy ionizing radiation from a $^{137}$Cs source and killed 4h later. Metallothionein levels were measured by $^{109}$Cd-hemoglobin saturation assay in the marrow (ng MT/mg protein) and liver (µg MT x $10^{-2}$/mg protein). Data reported are the means of 3 determinations, SEM = 2 for marrow and SEM = 11 for liver. * indicates a statistical difference from non-irradiated control rats (p<0.05).
Table 5-2

Cu,Zn superoxide dismutase and blood parameters following radiation exposure.

<table>
<thead>
<tr>
<th></th>
<th>Body Wt. (g)</th>
<th>Plasma Zn (µg Zn/ml)</th>
<th>Hct. %</th>
<th>Cu,Zn SOD U/mg pro.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>230</td>
<td>1.9</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td>0.1 Gy</td>
<td>133</td>
<td>1.7</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>0.25 Gy</td>
<td>132</td>
<td>2.0</td>
<td>36</td>
<td>33</td>
</tr>
<tr>
<td>0.5 Gy</td>
<td>142</td>
<td>2.1</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>1.0 Gy</td>
<td>127</td>
<td>1.8</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>5.0 Gy</td>
<td>148</td>
<td>1.9</td>
<td>36</td>
<td>23</td>
</tr>
<tr>
<td>SEM</td>
<td>5.32</td>
<td>0.03</td>
<td>1.34</td>
<td>3.1</td>
</tr>
<tr>
<td>0h</td>
<td>147</td>
<td>1.9</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>2h</td>
<td>138</td>
<td>1.8</td>
<td>37</td>
<td>21</td>
</tr>
<tr>
<td>4h</td>
<td>132</td>
<td>1.7</td>
<td>36</td>
<td>16</td>
</tr>
<tr>
<td>8h</td>
<td>144</td>
<td>1.5</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>24h</td>
<td>146</td>
<td>1.7</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>SEM</td>
<td>6.2</td>
<td>0.05</td>
<td>1.22</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Rats were exposed to 0, 0.1, 0.25, 0.5, 1.0 or 5.0 Gy radiation and killed 4h later. Marrow Cu,Zn SOD activity (units/mg protein) and blood parameters were measured. Another group of rats were exposed to 0.25 Gy radiation and killed at the indicated times. Control rats were placed in the radiation chamber, but not exposed to the radiation source. Data represent the means of three determinations. No statistically significant differences were determined from these data.
the results are shown in Figure 5-8. In the liver, metallothionein concentrations reached their highest point (1.5 ± 0.4 µg MT/mg protein) four hours after radiation exposure. However, marrow metallothionein levels are highest (76 ng MT/mg protein) eight hours after irradiation. Marrow metallothionein concentrations four and eight hours after 0.25 Gy exposure reached maximums of 87 and 93 ng MT/mg protein, almost two-fold higher in comparison to 53 ng MT/mg protein for non-irradiated control rats. The overall average of marrow metallothionein over time following 0.25 Gy exposure was statistically significant (p<0.05) compared to control rats (67 ng MT/mg protein and 56 ng MT/mg protein).

The graph in Figure 5-9, compares marrow and liver metallothionein concentrations in the irradiated group to the non-irradiated control group. The latter group was subjected to the same stress conditions of traveling to and from the Health Science Center, but were not irradiated. Metallothionein concentrations in the liver of the control rats closely parallel the time response of metallothionein to radiation exposure. These data suggest that the liver response to 0.25 Gy ionizing radiation is simply a response associated with stress. In contrast, this control group did not show any increase in marrow metallothionein, suggesting marrow does not exhibit short term effects of stress.
Figure 5-8. Time-course response of metallothionein to 0.25 Gy radiation. Rats were exposed to 0.25 Gy ionizing radiation from a $^{137}$Cs source and killed 1h, 2h, 4h, 8h and 24h later. Metallothionein levels were measured by $^{109}$Cd-hemoglobin saturation assay in the marrow (ng MT/mg protein) and liver ($\mu$g MT x $10^{-2}$/mg protein). Data reported are the means of 3 determinations, SEM = 4 for marrow and SEM = 10 for liver. * indicates a statistical difference from 1h (p<0.05).
200

Marrow

154

Liver

0

--

ca

0

1

2

4

8

24

Hours after Irradiation

Metallothionein

50

100

150

200

Marrow

Liver
Figure 5-9. Comparison of metallothionein levels over time between irradiated and non-irradiated controls. Irradiated rats were exposed to 0.25 Gy ionizing radiation from a $^{137}$Cs source and killed 1h, 2h, 4h, 8h and 24h later. Non-irradiated control rats were exposed the stress of travel and placed in the radiation chamber, but not exposed to the $^{137}$Cs source and killed 1h, 2h, 4h, 8h and 24h later. Metallothionein levels were measured by $^{109}$Cd-hemoglobin saturation assay in the marrow (ng MT/mg protein) and liver (µg MT x 10$^2$/mg protein). Data reported are the means of 3 determinations. * indicates a statistical difference from non-irradiated controls determined by the two-tailed Student's unpaired t-test (p<0.05).
A. Marrow and Liver metallothionein levels after irradiation at 0.25 Gy.

B. Marrow and Liver metallothionein levels in control conditions.

Hours After Irradiation at 0.25 Gy
Superoxide dismutase activity in the bone marrow of rats exposed to 0.25 Gy radiation was maximal within two hours of exposure (Table 5-2). Lower overall SOD activity was measured in this experiment, compared to the dose-response experiment. However, again, a two-fold increase in Cu,Zn superoxide dismutase activity was measured compared to the control non-irradiated group. None of these differences were statistically significant.

Discussion

Together these studies suggest that bone marrow metallothionein does not respond to some specific cytokines or radiation stress. However, as in the dietary experiment, metallothionein concentrations in the bone marrow respond most dramatically to the amount of zinc in the diet. It is likely that small changes found in response to the cytokines and radiation exposure may be related to altered hematologic parameters and the requirements of induced clonal expansion: proliferation and differentiation. While the response to dietary zinc may represent a general response by constitutive late stage progenitors already involved in protein and RNA synthesis for differentiation and function.

Both interleukin-1 and interleukin-6 have been associated with transient changes in hematologic parameters, making firm conclusions for or against cytokine induced metallothionein problematic. Within the time frame of our protocol, these cytokines should have had slightly differing
hematologic effects, based on the literature. Interleukin-6 should have provided an enriched population of intermediate and late stage normoblasts in the marrow, as well as an expansion of the myeloid cells (Ulich et al., 1991). On the other hand, interleukin-1 should have produced the same myeloid shift, without changing the erythroid compartment (Ulich et al., 1987).

Metallothionein concentrations were generally higher in the interleukin-1 treated group, compared to the interleukin-6 treated group. This is indirect evidence suggesting that the myeloid cells may respond to these cytokines by increasing metallothionein. Although significant increases in metallothionein were not found in this study, metallothionein induction has been seen in preliminary dot blots of total marrow RNA (Huber and Cousins, 1989). It should be noted that these preliminary results could not be confirmed by Northern blot analysis.

The increase in bone marrow metallothionein in response to low-doses of irradiation, may result from an increased demand for free radical scavengers in the surviving late stage progenitors. The tendency for metallothionein concentrations to decrease at higher radiation exposures, is the likely result of an increase in the number of BFU-e progenitors which have not begun to actively synthesize RNA or protein. These data lend further support that changes in
metallothionein concentrations in the bone marrow are occurring in only a fraction of the nucleated cells. The fact that no interaction was detected between dietary zinc and cytokine treatment in the bone marrow supports the idea that zinc and metallothionein levels in this tissue are tightly regulated. Cousins and Lee-Ambrose (1992) have shown a strong correlation between dietary zinc content and the amount of labeled $^{65}$Zn appearing within two hours in the bone marrow. Such increases in zinc flux are detectable in the bone marrow, but this could represent additional zinc which may not be necessary for cellular functions or even present within marrow cells. In any event, the data suggest that when dietary zinc status is compromised, a mechanism exists to provide the marrow with the requisite amount of zinc to maintain constitutive, zinc-dependent hematological functions. This would explain why hematological parameters are not readily changed during marginal zinc intakes.

The interaction of dietary zinc and cytokines in the liver suggests that the acute-phase response carried out by the liver may be compromised during marginal zinc intakes. These data confirm the findings by Huber and Cousins (1988) showing that marginal zinc status attenuated the response of metallothionein gene expression to interleukin-1α in the maternal liver. Recently other acute-phase reactants (acid glycoprotein and c-reactive protein) have been shown to have
sequences of significant homology to the metal regulatory element defined for metallothioneins (Yiangou et al., 1991), suggesting other proteins which may be affected during marginal zinc status.

There is not always a good correlation between metallothionein mRNA and metallothionein protein levels in response to these cytokines. For example, the ratio of metallothionein mRNA and metallothionein protein in the liver in response to interleukin-6 is not uniform in rats fed restricted, adequate or supplemental zinc intakes. This could be explained on a purely temporal basis. However, another explanation is that metallothionein may have been turning over more rapidly in the zinc deficient rats. Furthermore, the level of zinc in the diet may be important in stabilizing the metallothionein mRNA or the synthesized protein. Additionally, zinc may influence the interaction of the cytokine with its receptor or its cellular uptake. It has recently been proposed that a family of cell surface receptors, including those for individual cytokines, have a Zn$^{2+}$ binding site (zinc sandwich) which markedly increases receptor/ligand binding and physiological effects (Cunningham et al., 1991). These studies support this hypothesis, which, if correct, suggests that hepatic acute phase events induced by interleukin-6 are dependent upon dietary zinc for normal actions.
Although metallothionein gene expression has long been known to be transcriptionally regulated, the possibility of additional regulation is a current topic of intensive investigation. Metal-specific stabilization of metallothionein mRNA has been documented in chicken and mouse cells (De et al., 1991) and in human HepG2 cells (Sadhu and Gedamu, 1989). Further regulation by ongoing protein synthesis was also investigated, and data suggested that some protein synthesis inhibitors increased the rate of transcription of the avian metallothionein gene, while others stabilized metallothionein mRNA (McCormick et al., 1991). Our data suggest possible post-transcriptional regulation in rat hepatic metallothionein gene expression.

Our results confirm the differential response of the two metallothionein isoform genes in a tissue-specific manner as described by Cousins and Lee-Ambrose (1992). Both liver and bone marrow metallothionein-1 responded dramatically to the amount of dietary zinc intake in the present studies. Metallothionein-2 expression by the bone marrow was lacking, similarly to its expression in the intestine (Cousins and Lee-Ambrose, 1992). It is possible to speculate that either very large amounts of metal and/or separate factors (e.g. glucocorticoids) are required to activate the promoter for expression of the metallothionein-2 isoform. However, large amounts of MT-2 expression were measured in the liver in response to both interleukin-1 and
interleukin-6. Similarly, Schroeder and Cousins (1990) detected metallothionein-2 expression in the presence of dexamethasone and interleukin-6 in total RNA from primary hepatocytes. Other authors have described similar differential induction of the human metallothionein gene isoforms (Sadhu and Gedamu, 1988) and isoforms in rainbow trout fibroblasts (Zafarullah et al., 1990) by various metal salts.
CHAPTER 6
ZINC METABOLISM IN ERYTHROCYTIC DEVELOPMENT

These studies were initiated as a corollary to work with humans, suggesting that metallothionein concentrations of red blood cells may be an accurate index of zinc status (Grider et al., 1990). The purpose of these experiments was to investigate zinc metabolism and metallothionein in a particular sub-set of bone marrow cells, erythroid progenitors, in the intact rat bone marrow. Our hypothesis in these experiments was that metallothionein was synthesized in nucleated progenitor cells within the bone marrow in response to exposure to increased zinc concentrations.

We used two models to induce erythropoiesis: acute blood loss (Millar et al., 1970), and phenylhydrazine induced hemolytic anemia (Tanaka et al., 1985). In response to both acute blood loss and phenylhydrazine treatment, erythropoietin synthesis and release occurs, leading to a 3-fold increase in numbers of erythropoietin-sensitive CFU-e (late stage erythroid progenitors) in the bone marrow (Iscove and Guilbert, 1979). Whole-body irradiation, on the other hand, was shown to decrease the number of CFU-e present in the bone marrow, since actively cycling cells are most susceptible to lethal irradiation. The early
progenitor (BFU-e) population in the bone marrow was increased by irradiation treatment. Therefore, these treatments are tools to study changes in the late stage progenitor cell population.

Acute Blood Loss-Induced Erythropoiesis

As discussed earlier, acute blood loss selectively induces the number of CFU-e cells in the bone marrow. Therefore, these experiments are designed to examine changes in bone marrow zinc distribution and metallothionein following induction of erythropoiesis. Since, no specific hematologic parameters have been documented in response to zinc deficiency, it is likely that erythropoiesis is not readily impaired by zinc deficiency. However, the rate controlling enzyme in heme synthesis, delta aminolevulinic dehydratase, is a zinc-dependent enzyme. Additionally, increased amounts of metallothionein have been measured in circulating red cells of humans supplemented with zinc over time (Grider et al., 1990).

Specific Experimental Protocols

Male weanling rats (75-100g) were given free-access to food and water for a 7d acclimation period. Rats were randomly assigned to acute blood loss (ABL, n=7) or control (CONTROL, n=5) groups. Under sodium pentobarbital anesthesia, 3-5 ml of blood was removed by cardiac puncture through the skin from ABL rats to induce erythropoiesis.
Control rats were anesthetized and the heart was pierced through the skin without blood loss. At this time a 10 µCi \(^{65}\)Zn tracer (in 0.25 µl) was administered intravenously into the jugular vein (Dunn et al., 1988), and distribution of the dose was examined after 6h. Tissue samples were prepared and incorporation of \(^{65}\)Zn was measured by gamma spectrometry. The data are reported as % of recovered dose in a total organ to accommodate slight differences in actual administered dose. Organ weights were based on body composition data (Owen, 1982). Metallothionein concentrations in the liver and marrow were also measured in separate groups of rats not administered the radiotracer. Serum zinc values were also obtained for these rats.

Another group of rats were given free-access to AIN-76A purified diets containing either 5 mg Zn/kg diet (depleted) or 30 mg Zn/kg diet (adequate) for 25d, following a 7d acclimation period to the zinc adequate diet. On 26d, rats within each dietary treatment were randomly assigned to two groups: acute blood loss (ABL) or sham (CONTROL), following the operations, 10 µCi \(^{65}\)Zn was administered (i.p.). Rats were killed at 24h and 72h (n=3/diet/treatment/time) following tracer administration. Tissues were prepared and the incorporation of isotope was measured by gamma spectrometry, as described above.
Results

Distribution of whole-body zinc was measured in zinc-adequate rats using a $^{65}$Zn tracer 6h following acute blood loss induced erythropoiesis or a sham operation. As shown by Table 6-1, only the bone marrow and the whole blood of the ABL group had significantly more $^{65}$Zn than their sham operated controls. However, only $^{65}$Zn uptake by the liver was lower in the ABL group, with other organs having similar accumulation of the isotope.

Induction of erythropoiesis by acute blood loss did not alter serum zinc values 24h after treatment compared to the sham operated control group (1.4 ± 0.2 and 1.5 ± 0.2 µg Zn/ml). As shown in Figure 6-1, liver metallothionein concentrations increased significantly in the ABL group 24h following ABL compared to sham operated control rats, returning to baseline levels by 72h. Although, the bone marrow metallothionein concentrations were not statistically different between treatments, the ABL group tended to reflect an increase in metallothionein compared to the control group. Within three days, metallothionein levels were indistinguishable between the groups in both organs.

The next experiment compared the response of zinc depleted (-Zn) and zinc adequate (+Zn) rats to acute blood loss induced erythropoiesis. The distribution of $^{65}$Zn was measured 24h and 72h after the induction of erythropoiesis,
<table>
<thead>
<tr>
<th>Tissue</th>
<th>ABL (n=7)</th>
<th>Sham (n=5)</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow</td>
<td>15.5 ± 2\textsuperscript{a}</td>
<td>13.4 ± 2\textsuperscript{b}</td>
<td>increased</td>
</tr>
<tr>
<td>Liver</td>
<td>41.1 ± 10\textsuperscript{b}</td>
<td>48.1 ± 10\textsuperscript{a}</td>
<td>decreased</td>
</tr>
<tr>
<td>Bone</td>
<td>19.4 ± 3</td>
<td>17.6 ± 4</td>
<td>no change</td>
</tr>
<tr>
<td>Skin</td>
<td>8.4 ± 1</td>
<td>7.1 ± 2</td>
<td>no change</td>
</tr>
<tr>
<td>Intestine</td>
<td>10.3 ± 2</td>
<td>10.7 ± 2</td>
<td>no change</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.5 ± 1</td>
<td>4.9 ± 1</td>
<td>no change</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.19 ± 0.2</td>
<td>1.21 ± 0.1</td>
<td>no change</td>
</tr>
<tr>
<td>Thymus</td>
<td>1.06 ± 0.1</td>
<td>1.14 ± 0.4</td>
<td>no change</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.7 ± 3</td>
<td>2.31 ± 0.5</td>
<td>variable</td>
</tr>
</tbody>
</table>

Rats were administered a 10 µCi $^{65}$Zn tracer (i.v.) immediately following acute blood loss (ABL) or a sham-operation, as described under Specific Experimental Protocols in this chapter. Tissue distribution of $^{65}$Zn was measured by gamma spectrometry after 6h, and reported as percentage of recovered dose in a total organ. Organ weights were based on body composition data (Owen, 1982). Data represent the means ± standard deviation of 7 (ABL) and 5 (Sham) rats. Means having different superscripts were statistically different by the two-tailed unpaired Student's t-test.
Figure 6-1. Metallothionein levels 24h and 72h following induced erythropoiesis. Erythropoiesis was induced by removing 3-5 ml blood by cardiac puncture under anesthesia (ABL) from zinc adequate (+Zn) or zinc depleted (-Zn) rats. Metallothionein was measured by $^{109}$Cd-hemoglobin saturation assay in the marrow (ng MT/mg protein) and liver (µg MT/g tissue). Data represent the means of 3 determinations, SEM = 4 for marrow and SEM = 3 for liver. * indicates a statistical difference (p<0.05).
The diagram shows the levels of metallothionein in controls and ABL treatments at 24h and 72h.

- **24h**
  - **CONTROL**: 38
  - **ABL**: 56 (Marrow), 55 (Liver)

- **72h**
  - **CONTROL**: 34
  - **ABL**: 35 (Marrow), 31 (Liver)
using the same acute blood loss model as the previous study. Tissue levels of $^{65}$Zn were compared between +Zn groups and -Zn groups at 24h, expressed as percent of recovered dose/organ (Table 6-2).

Zinc-adequate rats from the first experiment responded to acute blood loss by increasing the amounts of $^{65}$Zn measured in the bone marrow and decreased uptake by the liver at 6h (Table 6-1). Table 6-2 shows that 24h after acute blood-loss, however, the +Zn rats had a markedly greater accumulation of the $^{65}$Zn dose in the skin and bone compared to that measured after 6h (Table 6-1). However, no difference in skin and bone uptake was measured between the ABL group and the sham group at 24h. Only the spleens of the zinc adequate group demonstrated the tendency to incorporate less $^{65}$Zn in the ABL group compared to the control group at 24h. However, by 72h after the tracer greater $^{65}$Zn accumulation was found in the skin of the +Zn ABL group compared to their control group (Table 6-3).

The zinc depleted (-Zn) group had several differences in $^{65}$Zn distribution at 24h compared to their sham operated control group. A significant increase in uptake of $^{65}$Zn by the bone marrow of the -Zn ABL group compared to its control group was found. Significantly less $^{65}$Zn was found in the intestine of the zinc-depleted ABL group at 24h compared the control group. Lesser amounts of the isotope were also
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Zinc Adequate 25d</th>
<th>Zinc Depleted 25d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABL (n=3)</td>
<td>Sham (n=3)</td>
</tr>
<tr>
<td>Blood</td>
<td>1 ± 0.2</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Marrow</td>
<td>13 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.7±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Intestine</td>
<td>11 ± 3</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>Liver</td>
<td>11 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Skin</td>
<td>18 ± 1</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Bone</td>
<td>36 ± 6</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Muscle</td>
<td>15 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.5±0.1</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2 ± 0.4</td>
<td>2 ± 0.2</td>
</tr>
</tbody>
</table>

Rats (6) were randomly assigned to dietary groups fed 30 mg Zn/kg diet (+Zn) or 5 mg Zn/kg diet (-Zn) for 25d. Rats from each dietary group underwent either an acute blood loss (ABL) or a sham (Sham) operation, and were administered 10 μCi $^{65}$Zn tracer (i.p.), as described under Specific Experimental Protocols in this chapter. Tissue distribution of $^{65}$Zn was measured by gamma spectrometry 24h after the operations, and reported as percentage of recovered dose in a total organ. Organ weights were based on body composition data (Owen, 1982). Data represent the means ± SD of 3 rats/diet/operation.
Table 6-3

Tissue distribution of $^{65}$Zn in zinc adequate and zinc depleted rats 72h following acute blood loss or sham operation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Zinc Adequate 25d</th>
<th>Zinc Depleted 25d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABL (n=3)</td>
<td>Sham (n=3)</td>
</tr>
<tr>
<td></td>
<td>ABL (n=3)</td>
<td>Sham (n=3)</td>
</tr>
<tr>
<td>Blood</td>
<td>1 ± 0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Marrow</td>
<td>8 ± 1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.5±0.1</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>Intestine</td>
<td>6 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Liver</td>
<td>6 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Skin</td>
<td>21 ± 4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Bone</td>
<td>37 ± 4</td>
<td>42 ± 7</td>
</tr>
<tr>
<td>Muscle</td>
<td>19 ± 4</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.4±0.1</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 ± 0.4</td>
<td>1 ± 0.2</td>
</tr>
</tbody>
</table>

Rats (6) were randomly assigned to dietary groups fed 30 mg Zn/kg diet (+Zn) or 5 mg Zn/kg diet (-Zn) for 25d. Rats from each dietary group underwent either an acute blood loss (ABL) or a sham (Sham) operation, and were administered 10 µCi $^{65}$Zn tracer (i.p.), as described under Specific Experimental Protocols in this chapter. Tissue distribution of $^{65}$Zn was measured by gamma spectrometry 72h after the operations, and reported as percentage of recovered dose in a total organ. Organ weights were based on body composition data (Owen, 1982). Data represent the means ± SD of 3 rats/diet/operation.
incorporated by the skin and bone in the zinc depleted group at 24h (Table 6-2), although these differences were not significant. However, within 72h significantly more $^{65}$Zn was found in the intestine of the -Zn ABL group compared to the control group, accounting for the largest percentage of the isotope remaining in this group (Table 6-3). In contrast, the largest amount of $^{65}$Zn measured in the -Zn control group was found in the skin (Table 6-3). The level of $^{65}$Zn in the bone marrow of the zinc-depleted ABL group was higher compared to the initial uptake at 6h by the zinc adequate ABL group (20% ± 2 and 16% ± 2), possibly suggesting that a certain amount of zinc is sequestered by this organ to replete zinc levels following the marginal dietary zinc feeding.

The whole body $^{65}$Zn distribution differed between zinc-depleted and zinc-adequate groups, regardless of treatment group. Isotopic dilution and deep-labeling of tissue zinc stores by turnover should have taken place by 72h. Irrespective of ABL or sham treatment, $^{65}$Zn levels in the blood, spleen, intestine and bone were quite different between the dietary groups. Much higher levels of $^{65}$Zn were measured in the blood, spleen and intestine, with lower levels in the bone of the depleted rats by three days, compared to the zinc adequate group (Table 6-3). There were also differences noted in the accumulation and distribution of $^{65}$Zn between CONTROL and ABL groups depending on dietary
group. The +Zn group had similar accumulation/turnover of tissue $^{65}$Zn after 72h, except in the bone and the skin. In contrast, significant differences in $^{65}$Zn accumulation and/or turnover were evident between the -Zn ABL and -Zn control group. Significantly more $^{65}$Zn accumulated in the spleen, intestine and liver of the -Zn ABL group, and less in the skin and bone compared to the -Zn control group.

**Phenylhydrazine-Induced Erythropoiesis**

These experiments used the phenylhydrazine-induced hemolytic anemia model as described by Tanaka et al. (1985). The anemia induces extramedullary erythropoiesis indicated by splenic enlargement and the active synthesis of CFU-e progenitors by the bone marrow. The purpose of this protocol was to selectively enrich a single population of cells in the bone marrow of the intact animal. Providing an acute dose of zinc to the anemic rats allows examination of the ability of these marrow cells to produce metallothionein.

**Specific Experimental Protocols**

Following the 7d acclimation period, rats were assigned to one of three groups: Control (Con), Phenylhydrazine-Saline (Phz-Sal) or Phenylhydrazine-Zinc (Phz-Zinc). The hemolytic anemia was induced by a series of injections (s.c.) of 60 mg phenylhydrazine/kg body weight/d. Injections were given on d1, d2 and d4, while d3 was a rest
day. Control rats were injected similarly with an equal volume of 0.9% saline (s.c.). An acute dose of 25 µmol zinc, as zinc chloride, was injected (s.c.) to the Phz-Zinc group at 0h; the other two groups were injected (s.c.) with an equal volume of 0.9% saline at 0h on the morning of d5. Rats were sacrificed 0h, 6h, 9h and 24h following the d5 injection. Plasma zinc, hematocrit, and metallothionein levels in the liver and bone marrow were determined.

Whole blood was collected by cardiac puncture in heparinized tubes to prevent clotting. The remaining blood was separated by centrifugation at 4500 xg for 15 min at 4°C. Residual red cells were removed by a second centrifugation a 2000 xg for 10 min at 4°C of the supernatant. Plasma zinc concentrations were measured by comparing air/acetylene flame atomic absorption spectrometry readings of a 1:5 sample dilution to readings of a standard curve using linear regression analysis. For some experiments, serum zinc was analyzed in the same manner, however, blood was allowed to clot on ice prior to the initial centrifugation. Aliquots were also removed in heparinized capillary tubes and immediately separated by centrifugation to measure hematocrit values.

In a subsequent experiment, all rats were made anemic by injection of phenylhydrazine to enrich the erythroblast population. As in the previously described experiment, 3 rats were given 25 µmol zinc or an equal volume of 0.9%
saline on d5. Bone marrow was harvested 24h later and pooled into two groups (+Zn) or (Control). The marrow was separated over a standard discontinuous Percoll gradient (40%, 70% and 80%) to obtain semi-purified cell fractions according to the procedure of Harrison et al., (1981). The separation gradients diluted from 100% Percoll (LKB-Pharmacia) contained 5 ml of each Percoll concentration.

The bone marrow plugs were dispersed to a single cell suspension in RPMI 1640 medium using 16 and 18 gauge needles. The cells were pelleted by centrifugation at 150 x g for 10 min and re-washed twice in LEMS media (0.3 M lactose, 14 mM 2-ß-ME, 2 mM EDTA and 150 mM NaCl) to prevent cell clumping. The pellet was resuspended in PBS buffer (1X PBS, 0.1% w/v CaCl₂, 0.1% w/v MgCl₂) and counted using a hemocytometer following a 1:1 dilution with trypan blue.

A cell suspension of approximately 10⁹ cells in 2 ml of PBS was gently layered over the Percoll gradient in a 30 ml glass Corex tube. The gradient was centrifuged 3000 xg for 30 min at 4°C. This density separation procedure yielded three enriched cell populations: erythroblasts (40%/70% interface), myeloid cells (70%/80% interface) and reticulocytes and debris in the pellet. The three populations were removed to clean tubes and diluted 20X with the PBS buffer and centrifuged at 300 xg to remove the Percoll. The pellets were resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.4) for metallothionein measurement.
Results

The phenylhydrazine treatment led to the expected dramatic increase in splenic weight, and a decrease in the hematocrit. However, body weight between the groups was not different. Table 6-4 shows the changes in weight and blood parameters between rats treated only with 0.9% saline (Con), anemic rats injected with 0.9% saline (Phz-Sal) and anemic rats administered 25 µmoles zinc (Phz-Zinc). Phenylhydrazine treatment alone, significantly decreased the serum zinc values at all time points, especially following the stress of the 0.9% saline injection on d5. Treatment with 25 µmoles zinc elevated the serum zinc value significantly through 9h, but these were reduced to control levels by 24h. Sera and liver homogenate cytosols from the phenylhydrazine treated rats showed signs of jaundice, indicating some liver damage and hemoglobinuria.

Although bone marrow metallothionein levels were initially quite high in the Phz-Sal group at 0h (96 ng MT/mg protein), this value tended to stabilize around 80 ng MT/mg protein throughout the rest of the time course. The means of this anemic group and the control group were not significantly different (p<0.05). Across all time points, there was a significant increase in the bone marrow metallothionein in the Phz-Zinc group compared to the Phz-Sal and the Con group (mean ± SD, n=20): 100 ± 40, 86 ± 28, 59 ± 24 ng MT/mg protein, respectively. Figure 6-2A
Table 6-4

Effect of phenylhydrazine treatment on serum zinc, body weight, spleen weight and hematocrit values.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Phz-Saline</th>
<th>Phz-Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Wt. (g)</strong></td>
<td>213 ± 10^a</td>
<td>188 ± 15^a</td>
<td>182 ± 12^a</td>
</tr>
<tr>
<td><strong>Spleen Wt. (g)</strong></td>
<td>0.62 ± 0.1^a</td>
<td>2.14 ± 0.1^b</td>
<td>2.05 ± 0.1^b</td>
</tr>
<tr>
<td><strong>Liver Wt./Spleen Wt.</strong></td>
<td>14</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Hct (%)</strong></td>
<td>36</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td><strong>Serum Zn 0h (µg/ml)</strong></td>
<td>1.6 ± 0.1^b</td>
<td>1.2 ± 0.1^c</td>
<td>2.0 ± 0.2^a</td>
</tr>
<tr>
<td><strong>Serum Zn 6h (µg/ml)</strong></td>
<td>1.6 ± 0.1^b</td>
<td>0.9 ± 0.1^c</td>
<td>2.5 ± 0.3^a</td>
</tr>
<tr>
<td><strong>Serum Zn 9h (µg/ml)</strong></td>
<td>1.2 ± 0.1^b</td>
<td>0.9 ± 0.1^b</td>
<td>2.3 ± 0.2^a</td>
</tr>
<tr>
<td><strong>Serum Zn 24h (µg/ml)</strong></td>
<td>1.6 ± 0.1^a</td>
<td>1.3 ± 0.1^b</td>
<td>1.9 ± 0.2^a</td>
</tr>
</tbody>
</table>

Rats were given 0.9% saline (s.c.) or 60 mg phenylhydrazine (Phz)/kg body weight (s.c.) on d1, d2, d4. On d5, control and Phz-Sal rats received 0.9% saline (s.c.), Phz-Zinc rats were given 25 µmol Zn (as ZnCl₂) at 0h. Serum zinc was measured by atomic absorption spectroscopy, and data are reported in µg Zn/ml. Values represent the means ± SD (n=4). Data were analyzed by one-way ANOVA and groups compared using Bonferoni's corrected p-value. Values within rows having different superscripts are significantly different (p<0.05).
compares the metallothionein concentrations in the bone marrow across treatments over the time course. Within 6h, the metallothionein levels in the marrow of the zinc treated rats increased, and remained higher than the other two groups.

Metallothionein levels in the liver of Con and Phz-Sal rats remained consistently close to baseline across the time course, indicating little induction by stress, associated with repeated handling over the treatment series. Liver metallothionein concentrations across all time points were (mean ± SD, n=14-16): 3.99 ± 2.8, 0.841 ± 0.22 and 0.783 ± 0.33 µg MT/mg protein in Phz-Zinc, Phz-Sal and Con groups, respectively. Figure 6-2B. illustrates the accumulation of metallothionein in the liver in response to the injection of the zinc salt, accumulating from initial levels of 1.1 ± 0.5 µg MT/mg protein at 0h to a peak of 6.3 ± 3.4 µg MT/mg protein by 9h.

When bone marrow plugs were removed, washed and separated over the discontinuous Percoll gradient, metallothionein concentrations could be measured in cell populations of similar densities. Cells were grouped in four densities: least dense cells in the upper quarter (damaged cells), cells from the 40/70% Percoll interface (erythroblasts), cells from the 70/80% interface (myeloid cells) and finally cells in the pellet (reticulocytes and
Figure 6-2. Metallothionein levels following phenylhydrazine induced erythropoiesis. Erythropoiesis was induced by a 4d series of phenylhydrazine injections (60 mg/kg/d (s.c.). Zinc (25 µmoles) or saline (0.9%) was injected on d5. Metallothionein levels were measured by the $^{109}\text{Cd}$-hemoglobin saturation assay 0h, 6h, 9h and 24h later in bone marrow (A.) and liver (B.). Data represent the means from 5-8 rats. * indicates a statistical difference (p<0.05), SEM = 12 for marrow and SEM = 0.5 for liver.
debris). Highest concentrations of metallothionein/mg protein were found in the erythroblast containing population, regardless of zinc pretreatment. The cells from the marrow pre-treated with zinc contained (119 ng MT/mg protein) overall compared to (52 ng MT/mg protein) for the control marrow. However, the distribution of metallothionein within the cell fractions was similar, regardless of zinc treatment. These results are in agreement with similar results reporting highest metallothionein-1 concentrations in the youngest reticulocyte fractions (Robertson et al., 1989).

Similarly, bone marrow cells obtained from rats maintained on a marginally zinc deficient (5 mg Zn/kg diet) or adequate (30 mg Zn/kg diet) diet for 25d were separated over the Percoll gradient. Regardless of dietary treatment, over 30% of the metallothionein was measured at the 40/70% interface, erythroblast fraction. However, the absolute amount of the protein cannot be compared directly, because of a dramatic difference in total protein between the zinc depleted and zinc adequate groups (6.9 mg and 2.4 mg protein). These protein differences would be expected during a zinc deficiency, and magnified by the removal of the structural proteins associated with the marrow plug.
Discussion

Using two models of in vivo erythropoiesis, these experiments have demonstrated for the first time changes in whole-body $^{65}$Zn distribution kinetics during erythropoiesis and changes in metallothionein concentrations in bone marrow containing an enriched population of late stage erythrocyte progenitor cells. These data support the contention that metallothionein concentrations in these cells are responsive to dietary zinc intake and status. Furthermore, this population of cells does not respond to stress by increasing metallothionein synthesis. The increase in marrow metallothionein in response to zinc, but not to phenylhydrazine treatment alone further supports these data. Therefore, the induction of erythropoiesis is not sufficient to alter marrow metallothionein levels, but may be driven by the zinc flux through the marrow.

Together these data suggest that the increased accumulation of $^{65}$Zn appearing in the bone marrow following induced erythropoiesis, correlates to a transient increase in metallothionein synthesis in this tissue. Within 72h, isotope concentration and metallothionein in the bone marrow returns to baseline, consistent with egress from the tissue. Although liver metallothionein concentrations also increase following induced erythropoiesis, this response is probably mediated by adrenal corticosteroids released during stress,
since no increase in zinc accumulation in this organ was measured.

Zinc deficiency altered the whole-body distribution of a $^{65}$Zn tracer, such that increased incorporation of the isotope was seen in the blood, liver, spleen and intestine and decreased incorporation was measured in the bone, regardless of induced erythropoiesis. These dramatic differences probably reflect differences in the mobilization rates of tissue zinc stores by the zinc depleted rats compared to the zinc adequate rats. Higher incorporation of the isotope would be expected in organs which had the lowest or most rapidly turning over zinc stores during the marginal zinc feeding period. Since zinc replacement was not necessary in these tissues in the zinc adequate rats, greater amounts of the isotope accumulated in the skin and bone.

However, the dietary zinc depletion tended to magnify and perhaps delay the differences in $^{65}$Zn distribution following acute blood loss induced erythropoiesis. The incorporation of $^{65}$Zn was 2-fold higher in the spleen of the ABL group by 72h, suggesting extramedullary erythropoiesis or migration of newly synthesized reticulocytes to the spleen. These data suggest that the isotope was not yet being uniformly incorporated in the zinc depleted rats, and was appearing in critically needed organs. The similar distribution of $^{65}$Zn in the zinc adequate rats (both ABL and
CONTROL) at 24h and 72h, suggests uniform incorporation and turnover such that zinc necessary for red blood cell replacement had been available by 24h.

The changes in $^{65}\text{Zn}$ distribution 6h following the operations, closely parallel other studies of kinetics at this time point, with respect the percentages of the isotope measured in each organ (Cousins and Leinart, 1988; Huber and Cousins, 1988). However, unlike the changes following the cytokine, interleukin-1, the increase in bone marrow uptake comes at the expense of liver uptake of $^{65}\text{Zn}$, rather than bone or skin, which incorporate a significant amount of the isotope. Distribution of the $^{65}\text{Zn}$ remaining at 24h in the zinc depleted group does reflect a similar change: decrease in bone, skin and intestine to compensate for the increase in bone marrow. The whole-body $^{65}\text{Zn}$ distribution data support a highly regulated system which is able to kinetically trap the requisite zinc, during a marginal dietary zinc deficiency. This is reflected by the ability of the bone marrow to increase $^{65}\text{Zn}$ uptake and metallothionein levels following acute blood loss induced erythropoiesis.

The tendency of all the phenylhydrazine-treated rats to have elevated levels of marrow metallothionein, regardless of zinc treatment, suggested that expansion in CFU-e population may require additional zinc and/or metallothionein. These data are consistent with the
increased zinc accumulation measured following acute blood loss in the previous experiments. Liver metallothionein measurements indicate that the zinc injection was sufficient to induce metallothionein synthesis, and that hemolytic anemia neither impairs nor enhances the ability of the liver to synthesize metallothionein.
CHAPTER 7
ZINC METABOLISM IN K562 CELLS

The K562 human erythroleukemic cell line, which can reversibly differentiate into normoblasts, was used as a model system to investigate the induction of metallothionein by zinc in the immature red blood cell. The intent of these investigations with K562 cells was three-fold: 1) to determine whether differentiation altered zinc metabolism or metallothionein expression, 2) to determine whether cytokines alter these parameters and 3) to determine if changes in metallothionein were proliferation dependent. Before these studies could begin, however, the manner in which K562 cells would respond to increased concentrations of zinc present in the growth media had to be characterized. Of specific interest was whether these cells possessed an active metallothionein gene, and would these cells accumulate significant concentrations cellular zinc in relation to the external supply.

Response to Medium Zinc Concentration

Specific Experimental Protocols

The response of the K562 cells to altered levels of medium zinc were conducted by incubating cells in 5, 15, 50 or 100 µM zinc for various time intervals. All cultures
were initiated with $2.5 \times 10^5$ cells/ml to insure log phase growth during the zinc exposure period. Basal RPMI 1640 medium had no measurable zinc (<1 µM), however the addition of 10% fetal calf serum brings the medium to 3-7 µM zinc, varying with the lot. Media zinc concentrations were adjusted using the addition of 35 mM zinc stock, as zinc sulfate. The 24h time point was selected for comparisons, since at this time metallothionein levels were detectable, and were the same as those measured at 48h. The response of metallothionein gene expression was measured at 0h, 1.5h, 3h, 4.5h and 6h after the addition of the metal based on previous data regarding the response of this gene to zinc in other cell systems. Metallothionein and cellular zinc data are presented on both a total cell protein and cell number basis. Considering that the cells are washed in an EDTA-containing buffer, it is assumed that cellular zinc is representative of intracellular zinc, including that associated with integral membrane proteins. Both manners of expression were chosen to account for cellular division during the incubation periods, as well as increases in protein synthesis.

Results

When K562 cells were incubated with medium zinc concentrations from 5 through 100 µM, metallothionein levels increased by 24h (Figure 7-1A), indicating that these cells
Figure 7-1. Response of K562 cells to increasing medium zinc concentrations. A. Metallothionein was measured in K562 cells incubated with 5, 50 or 100 µM zinc for 24h by the $^{109}$Cd-hemoglobin saturation method. Data represent the mean of 10-11 determinations from 3 separate experiments and are expressed on the basis of total cell protein (SEM = 0.2) and also cell number (SEM = 0.2). * indicates a statistical difference (p<0.01). B. Cellular zinc concentration was measured by atomic absorption spectroscopy in K562 cells incubated with 5, 15, 50 or 100 µM zinc for 24h. Data represent the mean of 10-12 determinations from 3 separate experiments, and are expressed on the basis of total cell protein (SEM = 0.3) and also cell number (SEM = 0.1). * indicates a statistical difference (p<0.01).
A.

![Graph A](image)

- Media Zinc Concentration (µM)
- µg MT/mg protein
- µg MT/million cells

B.

![Graph B](image)

- Media Zinc Concentration (µM)
- nmol Zn/mg protein
- nmol Zn/million cells
possess zinc-responsive metallothionein genes. However, metallothionein was barely detectable in cells incubated in 5 or 15 µM zinc medium (0.13 ± 0.04 µg/mg protein and 0.16 ± 0.05, respectively). Metallothionein concentrations increased two fold (0.249 ± 0.02 µg MT/mg protein) and thirty-three fold (4.3 ± 0.6 µg MT/mg protein) in 50 and 100 µM zinc containing medium, respectively. As expected, cellular zinc concentrations did not change significantly, except in the cells incubated in 100 µM zinc, as shown in Figure 7-1B.

Using the molecular weight of metallothionein, 6500 daltons, and its characteristic ability to bind 7 gram atoms zinc/mole metallothionein, the stoichiometric relationship between the increase of metallothionein and intracellular zinc can be calculated. Based upon this calculation, less than 10% of total intracellular zinc is bound to metallothionein in K562 cells incubated in 5 or 50 µM zinc medium, but increased to 36% when medium zinc is 100 µM. In other words, the cells incubated in the 5 µM medium had 0.02 nmoles MT, and this increased to 0.66 nmoles in cells from 100 µM zinc medium. The amount of intracellular zinc increased from 3.18 nmoles to 12.7 nmoles.

The Northern blot analysis (Figure 7-2) of K562 cells incubated in 50 or 100 µM from 0 to 6h, showed a visible increase in metallothionein mRNA by 3h regardless of the
Figure 7-2. Northern blot analysis of RNA from K562 cells in response to zinc for up to 6h. Total RNA was extracted from cells incubated in 50 or 100 µM and 4.6 µg RNA/lane was fractionated through 1.5% agarose. Hybridization was to [α-32P]-CTP random primer extension generated probes for MT-1 and β-actin cDNA.
level of zinc in the medium. Using densitometry units, and normalization to β-actin gene expression, significantly more metallothionein mRNA was transcribed in the K562 cells incubated in 100 µM zinc compared to 50 µM.

Metallothionein accumulation in response to 100 µM zinc was measured in a similar time course: after 0h, 3h, 6h, 9h, and 24h is illustrated in Figure 7-3. Increased metallothionein was detected within 3h (0.3 µg MT/mg protein) incubation with the exogenous zinc, reaching a statistically significant difference (p<0.05) from controls by 6h (3.4 µg MT/mg protein). By 9h (6.0 µg MT/mg protein), the accumulation appeared to maximize and was not different by 24h (6.3 µg MT/mg protein). These data agree with changes documented in gene expression (Figure 7-2).

Cytokine Response Studies

Specific Experimental Protocols

For these studies, growing K562 cells were resuspended at starting concentrations of 2.5 x 10^5 cells/ml in medium containing 50 µM zinc. These concentrations were necessary to drive cellular metallothionein levels into a measurable range. Simultaneous to the addition of zinc to the medium, a dose response curve was generated to rhIL-1α and rhIL-6. The interleukin-1 was added at 0, 100, 500 and 5000 D-10 units/ml medium. The interleukin-6 was added at 0, .25, 2.5
Figure 7-3. Metallothionein levels in K562 cells incubated in 100 µM zinc medium for up to 24h. Metallothionein levels were measured by the $^{109}$Cd-hemoglobin saturation method. Data represent the mean of 10-12 determinations from 3 separate experiments, and are expressed on the basis of total cell protein (SEM = 0.5) and also cell number (SEM = 0.1). * indicates a statistical difference (p<0.05).
Hours in 100 µM zinc medium

- **µg MT/mg protein**
- **µg MT/million cells**

<table>
<thead>
<tr>
<th>Time</th>
<th>µg MT/mg protein</th>
<th>µg MT/million cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>3.4</td>
<td>*</td>
</tr>
<tr>
<td>9</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>24</td>
<td>6.3</td>
<td>*</td>
</tr>
</tbody>
</table>

* indicates significant differences.
and 12.5 D-10 units/ml medium. These concentrations were selected based on studies in primary rat hepatocytes (Schroeder and Cousins, 1990). Total RNA was extracted following 6h incubation, and metallothionein levels were measured after 24h, as described in Chapter 3.

Results

The K562 cells failed to respond to either interleukin-1α or interleukin-6 by inducing metallothionein mRNA or synthesizing metallothionein protein. As mentioned above, both control and cytokine treated cells were incubated in 50 µM zinc medium, in order to drive metallothionein into a measurable range. After 6h incubation with 0, 100, 500 or 5000 units rhIL-1α/ml medium or 0, 0.25, 2.5 or 12.5 units rhIL-6/ml medium, total RNA was extracted and subjected to northern blot analysis. Figure 7-4 provides evidence that regardless of the amount of cytokine, no change was seen in metallothionein gene expression. Similarly, metallothionein protein levels after 24h were not different between treatments. Metallothionein concentrations (mean ± SD; n=6-8) were 0.46 ± 0.05 µg MT/mg protein, 0.53 ± 0.04, 0.51 ± 0.03 and 0.48 ± 0.03 at zero, low, medium and high rhIL-1α concentrations. Similar concentrations were found in K562 cells treated with 50 µM zinc and interleukin-6: 0.49 ± 0.04 µg MT/mg protein, 0.48 ± 0.04, 0.54 ± 0.05 and 0.50 ± 0.03.
Figure 7-4. Northern blot analysis of RNA from K562 cells in response to interleukin-1 or interleukin-6. K562 cells were incubated in 50 µM zinc medium and 0, 100, 500 or 5000 Units IL-1/ml or .25, 2.5 or 12.5 Units IL-6/ml for 6h. Total RNA was extracted and 4.6 µg RNA/lane was fractionated through 1.5% agarose. Hybridization was to [α-35P]-CTP random primer extension generated probes for MT-1 and β-actin cDNA.
Hemin-Induced Differentiation Studies

Specific Experimental Protocols

The response of metallothionein following differentiation was investigated using the hemoglobin degradation compound, hemin. K562 cells, at initial concentrations of $2.5 \times 10^5$ cells/ml, were induced to differentiate by the addition of 80 $\mu$M hemin. These parameters were selected based on the literature (Tomoda et al., 1991), which suggested that by 48h, greater than 60% of the cells would be synthesizing fetal hemoglobins. In addition to the hemin addition, media contained 5, 50, or 100 $\mu$M zinc. Duplicate control cultures were used for comparisons and contained no hemin. Metallothionein and intracellular zinc concentrations were measured after 48h. Metallothionein gene expression was assessed 0h, 3h, 6h, 24h, and 48h following the addition of 100 $\mu$M zinc, and 100 $\mu$M zinc plus 80 $\mu$M hemin.

Results

When K562 cells were induced to differentiate, as evidenced by hemoglobin synthesis, in 50 or 100 $\mu$M zinc medium, metallothionein accumulation was decreased compared to control cultures. Figure 7-5A illustrates that control cultures responded characteristically at 48h to the level of zinc in the medium: (mean ± SD) 0.2 $\mu$g MT/mg protein ± 0.1 in the 50 $\mu$M zinc cultures, and 7.9 $\mu$g MT/mg protein ± 1.9
Figure 7-5. Response of K562 cells to hemin induced differentiation. K562 cells were incubated in 50 or 100 µM zinc containing medium with (Hemin) or without (Control) 80 µM hemin for 48h. A. Metallothionein was measured in K562 cells by the \(^{109}\text{Cd}-\text{hemoglobin saturation method. Data represent the mean of 10-11 determinations from 3 separate experiments and are expressed on the basis of total cell protein (SEM = 0.3) and also cell number (SEM = 0.2). * indicates a statistical difference (p<0.01). B. Cellular zinc concentration was measured by atomic absorption spectroscopy in K562 cells. Data represent the mean of 10-12 determinations from 3 separate experiments and are expressed on the basis of total cell protein (SEM = 0.4) and also cell number (SEM = 0.2). * indicates a statistical difference (p<0.01).
A.

- **50 μM Zn**
  - Control: 0.5 μg MT/mg protein, 0.1 μg MT/million cells
  - Hemin: 0.3 μg MT/mg protein, 0.0 μg MT/million cells
  - Control: 7.9 μg MT/mg protein, 2.6 μg MT/million cells
  - Hemin: 3.3 μg MT/mg protein, 1.4 μg MT/million cells

- **100 μM Zn**
  - Control: 15.2 nmol Zn/mg protein, 2.5 nmol Zn/million cells
  - Hemin: 12.5 nmol Zn/mg protein, 2.3 nmol Zn/million cells
  - Control: 15.2 nmol Zn/mg protein, 1.9 nmol Zn/million cells
  - Hemin: 15.2 nmol Zn/mg protein, 1.5 nmol Zn/million cells

B.

- **50 μM Zn**
  - Control: 50 μg MT/mg protein, 50 μg MT/million cells
  - Hemin: 100 μg MT/mg protein, 100 μg MT/million cells

- **100 μM Zn**
  - Control: 12.5 nmol Zn/mg protein, 15.2 nmol Zn/million cells
  - Hemin: 12.5 nmol Zn/mg protein, 15.2 nmol Zn/million cells
when medium zinc was 100 µM. Hemin-induced cultures had reduced metallothionein accumulation by 48h: 0.1 ± 0.03 µg MT/mg protein and 3.2 ± 0.9, respectively. The 60% decrease in metallothionein in the hemin-treated cultures in 100 µM zinc was statistically significant (p<0.01). Although the difference at 50 µM zinc was not significantly different, the same trend is observed. Intracellular zinc concentration was not different between control and hemin-treated cultures (Figure 7-5B). However, higher amounts of intracellular zinc were measured in cells from 100 µM zinc.

To investigate whether the decreased amount of metallothionein occurred by reduced metallothionein expression was examined early in the heroin-treatment response. The Northern blot in Figure 7-6 shows that metallothionein mRNA levels are only slightly decreased in the hemin-treated cultures, compared to the controls at the 3h and 6h time points. When MT mRNA is compared at 24h or 48h, no visible difference was detected between cultures. These slight differences, then, could not account for the 60% reduction in metallothionein accumulation in the 100 µM zinc cultures.
**Figure 7-6.** Northern blot analysis of RNA from K562 cells in 100 µM zinc ± 80 µM hemin. K562 cells were incubated in 50 or 100 µM zinc containing medium with (+) or without (−) 80 µM hemin for 0, 3 or 6h. Total RNA was extracted and 4.6 µg RNA/lane was fractionated through 1.5% agarose. Hybridization was to [α-³²P]-CTP random primer extension generated probes for MT-1 and β-actin cDNA.
Mitomycin-C Anti-Proliferation Studies

Specific Experimental Protocols

Proliferation studies were similarly conducted. Growing cells were resuspended in RPMI 1640 (10%FCS) culture medium and incubated for 2.5h with 25 µg mitomycin-c/ml medium to impair DNA replication. After washing cells three times to remove the mitomycin-c, cells were counted and resuspended in fresh medium containing 100 µM zinc at 2.5 x 10^5 cells/ml, and incubated 24h free of the anti-proliferative agent. Metallothionein levels were measured after 24h, and MT mRNA was measured at 0h, 6h and 24h.

Results

The anti-proliferative agent (mitomycin-c) was used to determine whether metallothionein synthesis seen with addition of 100 µM zinc to the medium was linked to cellular proliferation in these cells. Overall protein synthesis in the mitomycin-c treated cells is not significantly reduced compared to the control cells, indicating that the ability of the cell to synthesize protein is not compromised by the anti-proliferative agent. However, Figure 7-7 shows that when K562 cells are pre-treated for 2.5h with mitomycin-c to block cellular proliferation, the synthesis of metallothionein over the following 24h is impaired. Control cells were not exposed to any level of mitomycin-c, and characteristically responded by doubling their cell number,
Figure 7-7. Metallothionein levels in K562 cells incubated in 100 μM zinc 24h following mitomycin-c treatment. K562 cells were pre-incubated with 0, 3.125, 6.25, 12.5 or 25 μg mitomycin-c for 2.5h, washed three times in 1X PBS and resuspended at 2.5 x 10^5 cells/ml in fresh 100 μM zinc media for 24h. Metallothionein was measured by the ¹⁰⁹Cd-hemoglobin saturation method. Data represent the mean of 12 determinations from 3 separate experiments and are expressed on the basis of total cell protein (SEM = 0.4) and cell number (SEM = 0.2). Means having different superscripts are statistically different (p<0.01).
and accumulating 4.4 µg MT/mg protein. In contrast, cells incubated with 6.25 µg or higher mitomycin-c, showed significantly reduced metallothionein accumulation in response to 100 µM zinc for 24h.

These data were substantiated by measurements of metallothionein gene expression. Figure 7-8 shows that these cells were able to synthesize characteristic levels of β-actin following mitomycin-c treatment, but metallothionein mRNA was much reduced compared to control cultures.

**Discussion**

The purpose of these experiments was to characterize the zinc induction of metallothionein in erythroid progenitor cells, using a tissue culture model. The K562 cell line provided evidence at the cellular level that cells which resemble the normal late stage progenitors (CFU-e) of the bone marrow, possess active and zinc responsive metallothionein genes. These cells did not respond to two cytokines: interleukin-1 and interleukin-6, however. When hemoglobinization was induced, these cells rapidly lost the ability to accumulate metallothionein in response to zinc, although metallothionein gene expression continued unchanged. Studies with mitomycin-c suggest that the increased accumulation of metallothionein may be linked to proliferation.
**Figure 7-8.** Northern blot analysis of RNA from K562 cells in 100 µM zinc for 6h after pre-incubation with mitomycin-c. K562 cells were pre-treated with 25 µg mitomycin-c for 2.5h, washed 3x with 1X PBS and resuspended in fresh medium containing 100 µM zinc for 6h. Total RNA was extracted and 4.6 µg RNA/lane was fractionated through 1.5% agarose. Hybridization was to [α-32P]-CTP random primer extension generated probes for MT-1 and β-actin cDNA.
Incubation with a very high zinc concentration (100 µM) caused over a thirty fold increase in metallothionein concentrations within 24h. Further accumulation was not measured at 48h, suggesting that turnover over of initially synthesized protein may have occurred. These changes were consistent with changes in metallothionein gene expression in the K562 cells.

The decrease in metallothionein in hemin treated cultures is of particular interest, since no reduction in metallothionein transcription was measured. These data could be explained by an increased turnover of the metallothionein in the hemoglobin synthesizing cells. Another possibility is that the cells actively producing hemoglobin (50%-60% of the hemin-treated cultures) are unable to synthesize metallothionein. However, this would be unlikely, since the oxidant state of the cells at this time would be high. It is most likely that zinc is being lost from the protein for other critical cellular functions, rendering metallothionein susceptible to increased proteolysis (Feldman et al., 1978).

Another explanation links metallothionein synthesis and accumulation to cellular proliferation. Our data demonstrate that in the absence of proliferation, the K562 cells also lose their ability to synthesize or accumulate metallothionein in response to zinc. There are many enzymes involved in DNA synthesis and cellular division which are
known to require zinc for activity (Vallee and Galdes, 1984). It is likely, that during the cellular division process zinc, possibly derived from or through metallothionein, is utilized in this manner.

The inability of either cytokine to induce metallothionein was surprising. Interleukin-1 had been shown to effect the expansion of myeloid cells (Ulich et al., 1989), and interleukin-6 to increase the numbers of late stage normoblasts (Ulich et al., 1990). We hypothesized that these signaling proteins would effect changes in metallothionein in these cells. In addition, other authors have postulated regulatory roles for these cytokines during inducible erythropoiesis (Johnson, 1990). Our data suggest that the metallothionein genes are responding directly to the amount of zinc in the medium, rather than through a change in other second messenger systems involving these specific cytokines.

We verified the bioactivity of the cytokine proteins, and found that the same preparations were also able to produce changes in IRD-98 cells (data not shown). Although K562 cells have been listed to contain receptors for interleukin-6 (Snyers et al., 1989), there have been no published investigations of changes in K562 cells following interleukin-1 or interleukin-6 treatment. However, following hemin-induced erythroid differentiation K562 cells have been shown to become resistant to tumor necrosis
factor α (Li et al., 1989). We can only conclude, then, that these cytokines were unable to induce metallothionein in K562 cells.

In conclusion, these experiments provide baseline responses of metallothionein to zinc, cytokines, erythroid differentiation and cellular proliferation. These data suggest that the K562 cell line may be a valuable tool to assess the roles of zinc and metallothionein during cellular processes, as well as erythroid development and regulation. These data provide indirect evidence that metallothionein levels in red blood cells may be an accurate way to titer zinc status, if these cells do not respond to other physiological mediators of stress.
CHAPTER 8
SUMMARY AND CONCLUSIONS

Zinc metabolism and metallothionein gene expression in the rat bone marrow were investigated under varying dietary zinc status, cytokine administration and ionizing radiation injury. Additionally, roles for zinc and/or metallothionein in the maturation of the erythrocyte lineage were explored by in vivo induction of erythropoiesis and in vitro differentiation of the erythroleukemic K562 cell line. To our knowledge these are the first studies to measure metallothionein and to examine roles for zinc in the stem cell progenitors of the bone marrow. However, studies have measured the functional responsiveness of terminally differentiated blood cells to depleted and supplemental levels of zinc, as described in Chapter 2. Increased metallothionein levels have been measured in erythrocyte lysates in response to several stimuli (Grider et al., 1990; Robertson et al., 1989; Tanaka et al., 1986).

Our first objective was to confirm the presence of metallothionein in the bone marrow and its responsiveness to zinc. Chromatographic separation of bone marrow cytosol, indirect protein assay and visualization of metallothionein mRNA confirmed the presence of metallothionein in the bone marrow. Metallothionein levels could be increased 2-fold by
injection of large amounts of zinc salts. Although, continuous infusion of the zinc salt by mini-osmotic pump did not continue to increase metallothionein in the bone marrow; dexamethasone tended to lower metallothionein concentrations in the bone marrow. Under conditions where rats were fed marginally deficient, adequate or supplemental zinc intakes for 14d, bone marrow metallothionein and zinc concentrations were susceptible to zinc depletion. However, additional zinc in the diet did not increase tissue zinc concentrations or metallothionein levels in the bone marrow.

The postulated immunoregulatory role of zinc and/or metallothionein was investigated using the cytokines interleukin-1 and interleukin-6, agents known to orchestrate broad systemic affects known as the acute-phase response. When rats were fed three levels of zinc intake for 14d prior to administration of interleukin-1 or interleukin-6, only the liver showed a significant interaction between dietary zinc status and cytokine treatment. The levels of zinc intake had primary control over marrow metallothionein concentrations, although interleukin-1 treated rats tended to show slight increases in metallothionein.

Measurements of metallothionein mRNA by Northern blot analysis demonstrated that metallothionein-1 was the primary gene isoform expressed by the bone marrow. Interleukin-1 induced metallothionein mRNA in the liver, regardless of the dietary zinc intake. However, interleukin-6 induction of
hepatic metallothionein mRNA was markedly linked to the dietary zinc intake. These data support the hypothesis that zinc may be required for the hepatic acute-phase response initiated through interleukin-6.

Metallothionein mRNA and metallothionein concentrations did not always correlate well. While this could be explained by temporal changes, zinc status may have altered metallothionein mRNA or protein stability. Firm conclusions regarding quantitative changes in metallothionein mRNA in the marrow are impossible given the technical difficulties encountered in these measurements. The small sample size of bone marrow available from one rat precluded measuring metallothionein and metallothionein mRNA within the same animal. Changes in the hematological cell distribution in response to the cytokines may also have contributed to this variability. Therefore, if one cell type responds by up-regulation of expression, and another type responds by down-regulation, then our results would be confounded. Variability in responses were seen between animals and obtaining undegraded mRNA from the marrow was problematic.

Tissue injury by ionizing radiation can also initiate a cytokine-mediated inflammatory response, whereby changes in metallothionein or whole-body zinc distribution may be important. Bone marrow metallothionein was measured following increasing doses of ionizing irradiation and in a
time-course following a low-dose of radiation (0.25 Gy). Irradiation exposure is known to decrease the number of CFU-e and other late stage progenitor cells in the bone marrow, leading to a proportional increase in the number of BFU-e progenitors remaining vital. Within 8h of 0.25 Gy radiation exposure, metallothionein levels in the marrow were 2-fold higher than levels in non-irradiated controls. However, as exposure dose increased, marrow metallothionein levels decreased. Superoxide dismutase levels in the bone marrow also doubled within 2h of exposure to low-dose irradiation and tended to decrease at exposures above 0.5 Gy.

The initial increase in metallothionein and superoxide dismutase may indicate a need for free radical scavengers for repair systems in damaged cells. As these cells die and are phagocytosed, lower metallothionein and SOD protein levels are measured. This can be attributed the killing of late stage progenitor cells in active cycle which were producing protein and messenger RNAs. Therefore, at higher radiation doses and at longer time intervals following irradiation, a proportional increase in early stage progenitors cells would be expected.

Zinc metabolism was investigated in the maturation of the erythrocyte lineage using acute blood loss and phenylhydrazine hemolytic anemia to induce erythropoiesis in vivo. These treatments have been shown to increase the
number of CFU-e (late stage progenitors) in the bone marrow. The kinetics of $^{65}$Zn distribution were measured 6h, 24h and 72h after acute bleeding and compared to sham operated controls. A comparison of whole-body $^{65}$Zn distribution was also made between zinc adequate and zinc depleted rats after acute bleeding. Initially, more $^{65}$Zn was found in the marrow and less in the liver of the bled group compared to the sham operated control group. In the zinc adequate rats increasing amounts of isotope were found in the skin and the bone, regardless of operation. By 72h, only the skin had higher and the bone had lower $^{65}$Zn in zinc adequate bled rats compared to sham-operated control rats. Metallothionein levels in the liver and marrow were higher in the acute blood loss group compared to the sham-operated controls at 24h.

Zinc depleted rats showed more profound and prolonged changes in $^{65}$Zn distribution between bled and sham-operated groups. More $^{65}$Zn was found in the marrow, but less isotope was found in the intestine, skin, bone and muscle of bled rats compared to sham-operated controls at 24h. The amount of $^{65}$Zn found in the liver was similar between groups. By 72h, significantly more isotope was found in spleen, intestine, and liver of the bled rats. Less $^{65}$Zn was found in the skin and bone compared to the sham-operated zinc depleted control group.
The $^{65}\text{Zn}$ distribution between the zinc adequate and the zinc depleted groups differed somewhat, regardless of the induction of erythropoiesis by acute blood loss. Less $^{65}\text{Zn}$ was found in the intestine, skin, bone, and muscle of the zinc depleted group 24h after the operations, which would be expected. A higher amount of $^{65}\text{Zn}$ was found in the liver and marrow of zinc depleted rats compared to zinc adequate rats, suggesting that these organs have an increased demand for the metal during periods of restricted zinc intake.

Phenylhydrazine hemolytic anemia was induced over 5d to induce erythropoiesis and enrich the marrow with CFU-e progenitors in active cycle. A single dose of zinc (25 $\mu\text{mol}$) was administered (i.p.) and marrow metallothionein levels were measured 0h, 6h, 9h and 24h later and compared to a saline-injected anemic control group, as well as a saline injected non-anemic control group. Metallothionein levels in the marrow of the zinc treated group were 2-fold higher than the non-anemic control group 6h and 9h later. The phenylhydrazine treated control group tended to have higher marrow metallothionein concentrations than the non-anemic control group, but metallothionein did not increase in a time-dependent manner in this group and did not achieve the increase of the zinc treated groups. Phenylhydrazine treatment alone was not sufficient to induce metallothionein in the liver.
Bone marrow cell suspensions were separated on standard discontinuous Percoll gradients to isolate cell types based on density. The banded interfaces were washed to remove the Percoll and metallothionein was measured in four cell populations: least dense damaged cells, erythroblasts at the 40/70% Percoll interface, myeloid cells at the 70/80% Percoll interface and the reticulocytes and debris in the pellet. The highest proportion of metallothionein was always measured in the cells of the 40/70% interface, regardless of the treatment.

Changes in zinc metabolism and metallothionein were examined at the cellular level in the erythroleukemic K562 cell line. This cell line, K562, possessed zinc responsive metallothionein genes. Although metallothionein and metallothionein mRNA were not measurable in K562 incubated in less than 50 µM zinc medium, doubling the zinc concentration (100 µM) increased metallothionein levels approximately 30-fold without compromising cell viability. Cellular zinc concentrations did not increase until medium zinc concentration was 100 µM. These cells did not increase metallothionein in response to either interleukin-1 or interleukin-6.

Differentiation along the erythrocyte lineage was accomplished by incubating K562 cells with the hemin for 48h. Simultaneous incubation with 50 or 100 µM zinc containing medium, led to similar metallothionein mRNA
expression compared to non-hemin treated control cells. However, metallothionein levels in hemin treated cultures were 60% lower than the non-hemin treated control cells. Mitomycin-c was used to inhibit normal DNA synthesis and proliferation of the K562 cells, after which the treated cells did not respond to 100 μM zinc medium by increased metallothionein production. Cellular protein synthesis was not significantly reduced in mitomycin-c treated cultures compared control cultures. Although β-actin mRNA synthesis was not altered by this treatment, metallothionein mRNA was significantly decreased in the non-proliferating mitomycin-c treated cultures.

This study presents evidence, for the first time, that the bone marrow possesses zinc responsive metallothionein genes. This organ is sensitive to zinc depletion under conditions where zinc intake is marginal. However, when zinc is available to the compromised system, increased amounts of the radiotracer, $^{65}$Zn, have been traced to the bone marrow in response to cytokines and the induction of erythropoiesis.

When increased levels of metallothionein were measured in response to zinc under all in vivo conditions, up-regulation was only 2-fold. This suggests that zinc concentrations in this tissue and/or the progenitor stem cells are tightly regulated and not able to accumulate. Future studies should be conducted to determine if this
regulation is at the level of the gene, or if sufficient zinc is unavailable to stabilize the metallothionein protein. Both the use of the mini-osmotic pumps and the phenylhydrazine model will be good models for these studies.

Although not explored deeply in this study, future studies regarding the radioprotective effect of zinc merit investigation. It seems likely that both zinc and/or metallothionein effect a cytoprotective role under these conditions. Studies examining clonal expansion of various cell lineages in the bone marrow of irradiated animals of varied zinc status would provide further clues regarding the role of zinc in these processes. Metallothionein levels could be quantitated following separation of marrow by Percoll gradients to determine whether specific cell populations responded with altered metallothionein levels.

The zinc response in the K562 cell line has been described, making more complex study designs possible. Our data suggest that metallothionein synthesis in these cells may be dependent on cellular proliferation. This should be examined further using additional agents which block proliferation at different sites. Additionally, studies can be done investigating zinc uptake, intracellular distribution and roles in erythrocyte maturation.
REFERENCES


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BIOGRAPHICAL SKETCH

Kirsten Lynn Huber was born in Baltimore, MD, on July 18, 1963, to Donald and Patricia Huber. She attended Liceo A-12 de Quillota, Chile, and James M. Bennett Sr. High School, receiving diplomas in 1980 and 1981, respectively. She graduated from Goucher College in Towson, MD, with a B.A. in Spanish in May, 1985. In 1986, she began studying under the guidance of Dr. Robert J. Cousins at the University of Florida, earning the M.S. degree in nutrition in August, 1988. In 1988, she was inducted into the Gamma Sigma Delta Honor Society, received the American Institute of Nutrition/Procter & Gamble award for excellence in original graduate student research, and was awarded the Pew Charitable Trust Fellowship to continue her study of zinc metabolism with Dr. Cousins. Kirsten married Robert Alan Speed, of Gainesville, FL, on June 30, 1990.

Kirsten has been afforded many opportunities to present her research findings at the annual American Institute of Nutrition meetings in the U.S., as well as a mini-symposium, Nutrition and Cytokines, sponsored by the prestigious Rank Prize Funds in the U.K. After completion of her doctorate, she will enter the University of Miami School of Medicine.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Cousins, Chairman
Boston Family Professor of Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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August, 1992

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