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LYSOSOMES IN THE IRON OVERLOAD GUINEA PIG MODEL:

A BIOCHEMICAL AND MORPHOLOGIC EVALUATION

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LYSOSOMES IN THE IRON OVERLOAD GUINEA PIG MODEL: A BIOCHEMICAL AND MORPHOLOGIC EVALUATION

By

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ABSTRACT

LYSOSOMES IN THE IRON OVERLOAD GUINEA PIG MODEL: A BIOCHEMICAL AND MORPHOLOGIC EVALUATION

By

E. Terence Adams

Lysosomal abnormalities have been associated with hepatic disease in iron overload of both humans and experimental animals. To investigate the prospect that cardiac lysosomes are altered in iron overload cardiomyopathy, we determined the free activity of selected myocardial and hepatic lysosomal enzymes in iron dextran treated guinea pigs (0.25, 0.5, 1.0 and 2.0 g Fe/kg BW). In addition, a serologic profile composed of serum glucosaminidase, serum iron, total iron binding capacity, and transferrin saturation was determined. Light and electron microscopic, as well as lipid peroxidation studies were included.

The free activity of hepatic glucosaminidase ($p<0.01$) and glucuronidase ($p<0.05$) and serum glucosaminidase activity ($p<0.01$) were significantly elevated at all levels of iron loading; free hepatic acid phosphatase ($p<0.01$) was increased at all but the lowest iron dose level. The free activity of myocardial glucosaminidase ($p<0.05$) was significantly increased at all iron dose levels when compared to pooled controls; free myocardial acid phosphatase was elevated only at the highest iron dose level of 2.0 g/kg ($p<0.05$).

Further studies demonstrated enhanced lipid peroxidation (as determined by the generation of malondialdehyde) in whole homogenates of liver and heart ($p<0.01$) at the iron dose level of 1.5 g Fe/kg.

Serologically, both serum iron and total iron binding capacity were significantly elevated at iron dose levels of 0.5 ($p<0.001$) and 1.0 g/kg ($p<0.05$ and $p<0.01$, respectively). Transferrin saturation was increased at 0.25 ($p<0.01$) and 1.0 g Fe/kg ($p<0.05$).

Morphologic studies indicated that intraperitoneal injection of iron dextran resulted in the initial deposition of iron particles within reticuloendothelial cells of the liver with subsequent hepatocellular iron deposition at higher iron loads. Myocardial iron particles were primarily located in connective tissue histiocytes and reticuloendothelial cells with rare evidence of deposition in cardiac myofibers. Ultrastructurally, these particles were aggregated in membrane-bound organelles consistent with lysosomes.

Additional preliminary studies indicated that iron loading (1.0 g Fe/kg) resulted in severe testicular atrophy and degeneration in male guinea pigs.

To Robert W. Leader, for unyielding support and

encouragement through arduous times

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TABLE OF CONTENTS

APPENDIX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APO_4ase ..... acid phosphatase

Df ..... desferrioxamine

Fe^{2+} ..... ferrous iron

Fe^{3+} ..... ferric iron

H_2O_2 ..... hydrogen peroxide

IHC ..... idiopathic hemochromatosis

IP ..... iron particles

$L^{\bullet}$ ..... lipid alkyl radical

LH ..... unsaturated fatty acid

$LO_2^{\bullet}$ ..... lipid peroxy radical

LOOH ..... lipid hydroperoxide

MDA ..... malondialdehyde

NAG ..... B-N-acetylglucosaminidase

O_2^{-} ..... superoxide radical

$OH^{\bullet}$ ..... hydroxyl radical

SI ..... serum iron

STEM ..... scanning transmission electron microscopy

Tf ..... transferrin

TIBC ..... total iron binding capacity

INTRODUCTION

INTRODUCTION

Primary and secondary iron overload disorders are characterized by variable degrees of cytosiderosis with the accumulation of iron in lysosomes of both reticuloendothelial and parenchymal cells of the liver, spleen, heart, pancreas and other tissues. As a consequence of severe siderosis a variety of clinical manifestations may evolve including hepatic cirrhosis, cardiomyopathy, endocrine pancreatic dysfunction, bronze skin pigmentation, arthropathy and hypogonadism.

The pathogenesis of iron-induced organ injury is poorly understood and is the subject of much debate. Previous research suggests that iron-mediated lysosomal disruption may play a role in initiating cell injury. Numerous reports demonstrate that hepatic lysosomes from iron overload patients are particularly fragile when compared to control subjects (1-4). Similar findings are documented in rats fed carbonyl iron (5,6), injected with a iron-sorbitol-citric acid complex (7) or iron nitrilotriacetate (8). Favored, mutually compatible, proposals for the mechanism of lysosomal membrane injury in iron overload include (a) accumulation of excess hemosiderin leading to physical disruption of the lysosome and (b) iron-catalyzed lipid peroxidation mediating the loss of lysosomal membrane integrity. Following lysosomal membrane injury, it is hypothesized that leakage of acid hydrolases results in destruction of intracellular constituents eventuating in cell damage (9)

Severe cardiomyopathy is a common clinical manifestation of hereditary and transfusion-induced iron overload. Chronically transfused thalassemic children with iron cardiomyopathy may develop severe clinical problems or even die during puberty. Investigation of this problem has been hampered by lack of an easily reproducible animal model that mirrors the human condition. Although iron overload has been reproduced in rats by injection of iron-sorbitol-citric acid complex or feeding carbonyl iron, these models are expensive and require 3 weeks - 12 months before the animals are iron loaded and studies can begin (5,10,11).

We have developed a previously unexplored animal model of iron overload. Guinea pigs intraperitoneally injected with iron dextran develop hemosiderosis of both their livers and hearts. This model was used to investigate the pathophysiology of iron-induced cardiac and hepatic toxicity.

In this study alterations in the stability of both myocardial and hepatic lysosomal membranes were examined. We determined the free activity of hepatic glucosaminidase, glucuronidase, and acid phosphatase as a measure of hepatic lysosomal fragility. Free myocardial acid phosphatase and glucosaminidase activity were evaluated as indicators of cardiac lysosomal fragility. In addition, the activity of serum glucosaminidase was determined in an effort to correlate the serum activity of this enzyme with tissue lysosomal enzyme changes.

In addition, we attempted to develop a serologic profile reflective of the iron status in the iron loaded guinea pig. We measured serum iron, total iron binding capacity and transferrin

saturation levels and subsequently compared values in this model with other mammalian species.

Morphologic studies were conducted to determine the distribution and location of iron in the heart and liver. Tissues were stained with hematoxylin/eosin and Prussian Blue for histologic evaluation. Ultrastructural studies were performed on both stained and unstained sections of left ventricular myocardium; selected sections were assessed by STEM analysis. A brief preliminary study was conducted to evaluate iron-induced testicular alterations in male guinea pigs.

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REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

Iron storage diseases in humans may be classified as either primary or secondary. Primary iron overload specifically refers to hereditary (idiopathic) hemochromatosis (IHC), the most common cause of significant iron overload. In this disease, increased intestinal absorption of iron on a diet with normal iron content, leads to iron overload with eventual tissue damage. Secondary iron overload defines a group of disorders (e.g., the thalassemia syndromes, transfusional hemosiderosis, African Siderosis) in which the iron overload is attributable to some abnormality other than a primary increase of intestinal iron absorption. Regardless of the cause, both primary and secondary iron overload disorders eventuate in a clinical syndrome of diabetes mellitus and pigmentary cirrhosis, often accompanied by cardiac dysfunction, gonadal failure and hyperpigmentation of the skin.

I. PRIMARY IRON OVERLOAD

Hereditary (idiopathic) Hemochromatosis (IHC)

The clinical syndrome of portal cirrhosis, diabetes mellitus, and bronze skin pigmentation was first described in 1865 by Trousseau (1). In 1871, Troisier (2) referred to a similar entity as "pigmentary cirrhosis in sugar diabetes" and "bronze diabetes." This was first referred to as hemochromatosis by von Recklinghausen in 1889 (3). The first major review and organization of the clinical data was published in 1935 by Sheldon (4), who recounted several instances of familial hemochromatosis and postulated that the disease was due to an inborn error of metabolism. MacDonald (5,6) later proposed the opposite

viewpoint, stating that most, if not all cases of idiopathic hemochromatosis were acquired. According to this theory, a toxic process, usually due to alcoholism, damages the liver and other organs and in some way predisposes this damaged tissue to accumulate excessive quantities of iron. Most recent investigations have supported the hypothesis that hereditary hemochromatosis is inherited, and it has been shown that the gene is linked to the major histocompatibility complex on the sixth human chromosome (7,8). Nevertheless, it is generally recognized that variable expressivity, as well as certain exogenous factors (e.g., alcohol intake), may influence the course and severity of the disease.

In IHC, increased amounts of iron are absorbed from the diet and deposited mainly in hepatocytes and in parenchymal cells of other organs. Increased iron deposition in reticuloendothelial cells, an important site of iron storage in normal individuals, usually does not occur in this disease until iron overload is far advanced (9).

The adult form of hereditary hemochromatosis is a disease which most often becomes clinically manifest during the fifth and sixth decade of life and is quite unusual before the age of 20 (Juvenile IHC appears clinically during the second and third decades of life). It is more common in men than in women. Persons who manifest clinically significant hemochromatosis undoubtedly have had progressive tissue iron overload for many years. The development of signs and symptoms of hemochromatosis is associated with cellular and tissue damage, thought to be due to toxic levels of iron. Involvement of the liver, heart, joints, pancreas, and other endocrine organs culminates in overt manifestations of the disease. The clinical course of the disease in

younger individuals seems to be more fulminant, (10,11) and cardiac dysfunction more common (12-14).

Linkage to HLA

A. Population Association

In 1975, Simon et al. (15) reported a relationship between hemochromatosis and the major histocompatibility (HLA) locus, noting an association with the HLA antigen A3. This discovery led eventually to the elucidation of the mode of inheritance. Walters et al. (16) and Shewan et al. (17) confirmed the findings of Simon and co-workers, and in addition noted an association with HLA-B7 among patients in Great Britain. However, the patients studied by Simon et al. (15,18) in Brittany, France more often possessed the combination of HLA-A3 with -B14. Simon also found that among unrelated patients with hereditary hemochromatosis the incidence of HLA-A3 was 78.4%, in contrast to an incidence of 27% among a group of apparently healthy adults. This apparent association is surprising because most diseases associated with a particular HLA antigen involve disorders of the immune system. Another unusual feature of this observation is that whereas most associations between HLA and disease involve B-series antigens, hemochromatosis appears to be more commonly associated with a A-series antigen. Class II antigens have also been implicated; a significant increase in DR2 was reported by Doran et al. (19), however the prevalence of several other Class II antigens (i. e., CW, Bf, DRw6, and GIo) has not been demonstrated to be significantly altered (20).

B. Antigen Association: True Significance

Current evidence indicates that hemochromatosis is associated with HLA-A3 more frequently than with B7 or B14. Doran et al. (19) reported an increased prevalence of A3 in B7-negative patients. In contrast to this evidence Valberg (21) suggested that HLA-B7 was more prevalent than A3 in hemochromatosis. Ritter et al. (22) reported a higher relative risk for B14 than A3. The conflicting evidence implies that A3, as well as B7 and B14 are all independent markers of the hemochromatosis allele. However, data on haplotype associations clearly indicate that haplotypes carrying B7 or B14 without A3 are not more frequent in hemochromatosis than in controls. However, haplotypes carrying A3 without B7 or A3 without B14, or also A3 without either B7 or B14 are significantly more frequent in hemochromatosis than in controls. Therefore, the significance of B7 and B14 as a marker of the hemochromatosis allele dissolves in the absence of A3; A3 remains the only independent marker. The presence of HLA-B7 and/or B14 alone is of no significance.

Simon et al. (18) was the first to suggest the possibility that the particular HLA antigens involved might not be critical and that a specific gene for hemochromatosis might be linked with the major histocompatibility complex on chromosome 6. The gene for this disease has now been mapped on chromosome 6, although its precise location remains a mystery. Numerous linkage studies (7,19,20,23-26) indicate the genetic distance between the HLA locus and hemochromatosis locus ranges from 0 to 2.5 centimorgans. Lalouel et al. (27) reported a distance of 1.0 centimorgan in a study involving 147 families.

II. SECONDARY IRON OVERLOAD

Secondary iron overload may result as a consequence of : a) anemia and ineffective erythropoiesis (due to long term red cell transfusions); b) liver disease, and c) excessively high intake of dietary iron.

A. Iron Overload Secondary to Anemias

Patients with iron loading anemias fall into two groups (a) those with decreased red cell production secondary to marrow failure (e. g., anemia secondary to marrow aplasia) whose major source of excess iron is blood transfusion, and (b) patients with hyperplastic bone marrow but ineffective erythropoiesis (e.g. thalassemia major). In the latter patients, the excess iron results not only from transfusion but also from increased iron absorption secondary to the ineffective erythropoiesis, thus the incidence of hepatic cirrhosis and cardiac involvement is much higher in this group. Patients with thalassemia major can be kept in good health for the first decade of life with regular blood transfusions. Inevitably, however, iron overload results with deposition of iron in many organs including the heart, liver and endocrine glands. The gross deposition of iron in the myocardium and conducting fibers causes arrhythmias and refractory cardiac failure, usually resulting in death between the ages of 16 and 22 years of age (28).

In thalassemia major, hepatic parenchymal iron deposition occurs even before transfusion therapy has begun; reticuloendothelial iron loading becomes prominent only after transfusions have been initiated (29). Even nonthalassemic patients chronically maintained on transfusion therapy for treatment of other anemic states may eventually

develop parenchymal cell iron overload and progress to a syndrome similar to hemochromatosis.

B. Iron Overload Secondary to Liver Disease

For the past two decades there has been confusion between IHC and alcoholic cirrhosis with increased stainable iron in the liver. It is now known that some stainable iron is quite common in normal subjects and in patients with cirrhosis.

Patients with alcoholic cirrhosis and stainable liver iron can be divided into two groups: (1) those patients who have a mild-to-moderate increase in stainable iron but relatively normal body iron stores, and (2) those patients with gross iron deposition and increased total body iron stores of the magnitude seen in IHC (15-50 g iron stores). The majority of the patients in the first group have alcoholic liver disease (usually cirrhosis) with some increase in stainable hepatic iron, but little increase in total body iron; these patients do not have IHC. Simon et al. (30) have concluded from their study, which included determination of HLA antigens, that such subjects are neither homozygous nor heterozygous for the disease. Liver iron concentration in such patients is usually less than twice the upper limit of normal (30,31). The reason for the increased stainable iron is unknown. It may be related in part to cell necrosis and the uptake of released iron by adjacent Kupffer and parenchymal cells.

The second group of alcoholic subjects who have cirrhosis with gross iron overload probably have IHC. A study of the hepatic pathology of alcoholic patients with a family history of IHC has revealed that in approximately 75% of them, the histologic changes are indistinguishable

from those in nonalcoholic subjects with IHC (32). The remaining 25% show very similar changes, but with features of alcoholic liver disease superimposed.

C. Iron Overload Secondary to Excessive Dietary Ingestion

Dietary iron overload, the prototype of which is African siderosis, has resulted in varying degrees of hemosiderosis.

African siderosis occurs in South African blacks and is one of the most thoroughly described examples of acquired iron overload. Initially, the excess iron deposits in reticuloendothelial cells (usually termed hemosiderosis). If the level of iron accumulation is sufficiently great, this syndrome may progress to a clinical picture resembling hemochromatosis, with iron deposition in hepatic parenchymal cells. Strachan first called attention to this entity in 1929 (33). Among 1100 autopsies performed in Johannesburg between 1924 and 1928, he found 33 instances of iron deposition comparable to that seen in hereditary hemochromatosis. Many of these individuals apparently had bronze diabetes mellitus and cirrhotic livers with large deposits of hemosiderin. A number of theories were proposed, but the etiology was not discovered until Walker and Arvidson (34) found that the prepared diet of these Africans contained approximately ten times more iron than could be measured in the uncooked food. Subsequently, it was demonstrated that iron derived from iron cooking pots was responsible for the excess iron in the diet. The traditional beer prepared by the Africans was brewed in these iron pots, and the amount of iron dissolved in the beer (approx. 4 mg Fe/dl) was enhanced by its lower pH. The distribution pattern of the resulting dietary iron overload differed

from IHC. In African siderosis, more hemosiderin was found in the spleen than in the liver. In contrast to IHC, hemosiderin was identified in the pancreas in only the severe cases (4). Hemosiderin deposits were unusually heavy in the bone marrow and in the duodenal and jejunal villi. Subsequently, distinct differences were recognized in the liver iron distribution; in African siderosis the hemosiderin was most prominent in the Kupffer cells. Only in the advanced stages was hemosiderin conspicuous in the hepatic parenchymal cells (35,36). In contrast, hemosiderin was prominent in the hepatic parenchymal cells in IHC, even in early stages of the disease. Thus, despite similar endstage disease manifestations, the clinical picture and the initial histologic distribution of the excess iron differs in these two conditions, suggesting different etiologic mechanisms. Clinically, the African siderotic deviates from the patient with IHC in that (1) the progress of the cirrhosis in the African is more rapid and is the principal cause of death, (2) there is a high incidence of porphyria cutanea tarda among African siderotics, and (3) cardiac complications are rare. (37).

III. MANIFESTATIONS OF IRON OVERLOAD IN SPECIFIC ORGAN SYSTEMS

The physical findings in patients with iron overload are usually related to involvement of the heart, liver, skin or testicle.

A. Heart

Cardiac failure is the most critical life-limiting complication of both primary and secondary iron overload. Cardiac dysfunction is diagnosed in 35% of patients with hereditary hemochromatosis;

approximately one third of untreated hemochromatotic patients die of cardiac failure or arrhythmia. Cardiac disease is especially common in younger patients, who often have a more rapidly progressive disease process.

The cardiac disease in iron overload is most likely due to iron deposition in heart muscle fibers. In an extensive study by Buja and Roberts (38) nineteen of 135 patients (four with idiopathic hemochromatosis and 131 with chronic anemia) had cardiac iron deposits (CID). The ventricular CID were grossly visible in nine patients and microscopically visible only in ten patients. Atrial CID were extensive in six patients with extensive ventricular CID, but in the other thirteen patients atrial CID were insignificant. Iron deposits in cardiac conduction tissue were minimal and always less than working myocardium. Of the nineteen patients with CID, three had idiopathic hemochromatosis; 16 had chronic anemia. Each anemic patient who received more than 100 units of blood had extensive CID unless chronic bleeding diatheses coexisted.

This study concluded that (1) grossly visible CID were always associated with cardiac dysfunction and usually chronic cardiac failure; (2) CID, usually extensive, occur in patients with idiopathic hemochromatosis; (3) extensive CID occur in patients who receive more than 100 units of blood unless bleeding diatheses coexist; (4) CID initially occur in ventricular myocardium, and were usually more extensive in ventricular than atrial myocardium; (5) CID were always more extensive in working than in conducting myocardium; (6) supraventricular arrhythmias correlate with the extent of CID in atrial

myocardium. These conclusions refuted the findings of a previous study (39).

Sanyal et al. (40) investigated both the histochemical and ultrastructural aspects of the heart in an adolescent with fatal congestive heart failure resulting from exogenous hemochromatosis. Histologic sections of myocardial tissue showed extensive iron deposits in all four chambers, papillary muscles and the conduction system; these were greatest within the outer third of the left and right ventricular myocardium. Iron deposits in the middle and inner third of the left ventricle were chiefly focal, in contrast to a more diffuse pattern in the outer third. There were no differences present between the other portions of the ventricular myocardium, however the interventricular septum contained heavy iron deposits. Degenerative changes of the ventricular myocardium were minimal and nonspecific, but occurred more frequently in the left ventricle.

Ultrastructural findings demonstrated that intracytoplasmic iron deposition followed one of three patterns: (1) paranuclear - a small collection of iron adjacent to the myofiber nucleus; (2) perinuclear - a more extensive deposition of iron around the nucleus; (3) diffuse - maximum quantities of iron present in a diffuse cytoplasmic pattern. It was noted that regardless of the pattern of deposition, some iron was consistently present within the nucleus and mitochondria. Another ultrastructural alteration of the myofibers was an increase in mitochondria associated with a decrease in myofibrils.

This study concluded that iron deposition in the ventricular myocardium follows a differential zonal pattern, with the greatest amount in the subepicardial region, an intermediate amount in the

subendocardial region and papillary muscles, and the least amount in the middle third of the ventricular myocardium. This zonal concept of iron distribution in ventricular muscles was originally suggested by Cappel et al. (41) and further supported by Buja and Roberts (38), as well as Olson et al. (42).

B. Liver

Manifestations of liver involvement are among the most constant features of iron overload disease (43); hepatomegaly is diagnosed in more than 90% of hemochromatotic patients. The enlarged liver usually is smooth rather than nodular, however hepatomegaly diminishes in many cases after phlebotomy therapy. Cirrhosis, when present, is of the micronodular type. If the disease remains untreated, cirrhosis will slowly progress to hepatic failure with all the associated complications. Treatment by venesection can reverse many or all of the abnormal histologic findings and architectural changes of cirrhosis (44-46). This observation supports the hypothesis that the cirrhosis is due to the toxic effects of excess iron. Recent reports indicate that the life expectancy of cirrhotic patients is markedly reduced compared to noncirrhotics, which had a life expectancy nearly identical to that of an age-matched normal population (47).

In the past, hepatoma occurred in about 14% (7 to 20%) of patients with hereditary hemochromatosis (12,39,48,49). Current evidence indicates that primary hepatocellular carcinoma and bile duct carcinoma are the leading causes of death in hemochromatotic patients (47).

C. Skin

Abnormal pigmentation is present in 90% of patients with fully expressed hemochromatosis. The color is variable, ranging from the classic bronze pigmentation to a slate gray appearance. The pigmentation may be patchy with dry, friable skin. The hyperpigmentation is due to the presence of melanin in the epidermis, however iron deposits may be found in the dermis, particularly around the sweat glands (50). This association with the sweat glands is not surprising, since iron is commonly deposited in other glandular organs of the body. The cause of the melanoderma is not known.

D. Gonads

Hypogonadism, impotence and loss of libido are prominent clinical features in men with IHC (51-56). Hypogonadism usually results from iron-induced damage of the pituitary gonadotrophs or the GnRH (gonadotropin releasing hormone) cells of the hypothalamus (57,58). The resulting FSH/LH (follicle stimulating hormone/luteinizing hormone) or GnRH deficiency leads to variable atrophy of seminiferous tubules and absence of spermatozoa and spermatids. Typically, the testes contain no excess iron or may have minimal deposits limited to blood vessel walls, thus sparing the germinal epithelium and interstitial cells (56). Bergeron and Kovacs (59) using a combination of immunocytochemical and Prussian Blue staining technique demonstrated that iron was preferentially localized in the gonadotropic cells of IHC patients. A minority of thyrotropic, corticotropic or somatotropic cells contained iron. Thus, panhypopituitarism with hypothyroidism and adrenal cortical insufficiency is a rare event in IHC (54). Previously, this form of

hypogonadism was considered irreversible and male patients were treated with testosterone supplementation (57). Recent case reports have indicated a restoration of normal pituitary and testicular function following aggressive phlebotomy therapy in hemochromatotic patients with hypogonadism (55,56)

It is notable that hypogonadism is particularly severe in both juvenile IHC and young well-transfused thalassemic patients.

IV. DIAGNOSIS

There is no single clinical sign which is pathognomonic of hemochromatosis, nor is there a single noninvasive test or combination of tests adequate for its diagnosis. In patients with various types of acquired iron overload, the underlying cause for the excess iron accumulation often is obvious, while in hereditary hemochromatosis there is no apparent inciting event. It is important to exclude causes of secondary iron overload in evaluating patients with suspected hemochromatosis.

A. Serum Ferritin

The most valuable test for the detection of tissue iron overload, regardless of location (parenchymal or reticuloendothelial), is measurement of serum ferritin. In healthy adults the concentration of ferritin in serum is directly related to the available storage iron in the body. The mean concentration of serum ferritin is three times higher in males than in females, with a range between 12 and 250 μg/liter (60,61). In patients with iron deficiency anemia concentrations are below 12 μg/liter, and in patients with iron overload the concentration may be as high as 10,000 μg/liter (60,62-64). A

retrospective study by Milder et al. (51) reported a mean serum ferritin level of 3,735 μg/liter (range 1,132-7,059 μg/liter) in a group of 34 hemochromatotic patients.

The measurement of mobilizable iron stores by quantitative phlebotomy in normal subjects show a good correlation with initial serum ferritin concentration (65) and a similar relation is seen in idiopathic hemochromatosis (66). In patients with transfusional siderosis the serum ferritin concentration is related to the amount of blood given (60,64), and there is a good correlation between serum ferritin concentration and the chemically determined iron concentration in liver biopsy tissue (64). Comparisons between serum ferritin concentration and the subjective semiquantitative visual assessment of stainable iron in bone marrow smears (63,67) show a crude relation between these two indexes of iron stores.

In most situations the serum ferritin concentration appears to reflect reticuloendothelial storage iron fairly accurately. Changes in reticuloendothelial iron are followed rapidly by changes in the serum concentration of ferritin. In normal subjects undergoing venesection the serum ferritin concentrations fall rapidly as iron stores are mobilized, and in both hemochromatosis and secondary iron overload, serum ferritin can be used to monitor therapeutic removal of excess storage iron. In addition, it has been used to evaluate the effect of chelation therapy in thalassemic patients with iron overload.

A limitation of serum ferritin concentration as a measure of iron stores is that it is not specific for iron overload; certain disease states unrelated to iron overload may also result in elevated serum ferritin levels (68). These include hepatocellular necrosis, malignant

neoplasia, leukemia and related disorders of the monocyte-macrophage system, and acute inflammation (68,69).

B. Transferrin Saturation

Transferrin saturation is the single most reliable screening test for hemochromatosis (70). Dadone et al. (26) demonstrated a transferrin saturation level above 62% to be the best indicator of homozygosity for hemochromatosis (92% correlation). However, other researchers have shown a cutoff of 55% to be the optimal discriminator between homozygotes and nonhomozygotes (70,71). Despite this the combination of serum transferrin and serum ferritin is still considered more sensitive than either test used alone.

C. Serum Iron Concentration

In primary hemochromatosis the serum iron concentration is generally increased; a mean value of 211.3 μg/dl was documented by Milder et al. (51). Serum iron has the disadvantage of being a nonspecific indicator of total body iron stores and may be markedly decreased during inflammatory processes (i.e., hypoferremia of inflammation).

D. Chelation Tests - Desferrioxamine Excretion

Parenchymal as opposed to reticuloendothelial iron overload may be further identified by chelation tests i.e., after intramuscular injection of the iron chelating agent desferrioxamine (500 mg), patients with hemochromatosis or other iron overload states (that maintain regular renal function and are ascorbic acid replete) usually will excrete substantially more iron in a 24 hour urine sample (> 2 mg) than

will normal subjects (72,73). The estimation of serum ferritin concentration, however, has now virtually replaced this test as an indication of excessive iron stores in the diagnosis of uncomplicated idiopathic hemochromatosis.

Another chelating agent employed in the assessment of iron overload is diethylenetriamine pentaacetic acid [DTPA] (74,75). The principles and techniques employed in the diagnostic use of desferrioxamine may be applied to DTPA, except the latter is given by i.v. infusion.

E. Hepatic Iron Stores

A definitive diagnosis of iron overload requires a liver biopsy. A percutaneous needle biopsy provides not only an estimation of tissue iron deposition, but also enables one to directly assess the distribution of iron among Kupffer cells and hepatocytes, as well as document associated changes such as fibrosis or cirrhosis. The extent of hepatic iron accumulation may be estimated semiquantitatively by histochemical grading. In a commonly used system (76), a grade of 0 signifies absence of iron, while grade 1, 2, 3, and 4 represent increasing amounts of stainable iron by Perl's Prussian Blue. Iron grades of 3 and 4 are common in untreated hemochromatosis. The iron deposition in hereditary hemochromatosis characteristically is greatest in the periphery of the lobules (32). Reticuloendothelial (Kupffer cell) iron may be inconspicuous despite significant hepatocellular involvement, although late in the disease process both types of cells may be involved. In secondary iron overload, iron accumulation usually is less, in the range of grade 1 and 2 (77). Reticuloendothelial iron

in a periportal distribution is prominent. Forms of severe secondary iron overload (i.e., advanced transfusional siderosis) result in markedly higher iron grades, thus may be histologically indistinguishable from advanced hereditary hemochromastosis.

Powell and Kerr (32) have further defined the criteria for histochemical grading. In this system, grade 4 is assigned to biopsies with stainable iron is virtually 100% of hepatocytes, grade 3 to biopsies with iron in 75%, and grades 2, 1, and 0 to correspondingly lesser degrees of hepatocyte involvement. Since stainable iron may be visualized even when tissue iron concentration is normal or only slightly increased, direct determinations of tissue iron content of liver biopsy specimens may be useful. Both histochemical and chemical iron determinations may be obtained conveniently using liver tissue from a single percutaneous needle biopsy (78,79).

F. Bone Marrow Iron

In normal individuals and in patients with secondary iron overload, iron stores may be judged by Prussian blue staining of bone marrow specimens and estimation of the ferritin and hemosiderin content of bone marrow macrophages; these estimates generally are well-correlated with RE iron elsewhere in the body (80). In hereditary hemochromatosis bone marrow iron often is inconspicuous despite iron overload in other organs (11,81-83). Thus, a comparison of marrow to liver iron content may be useful in differentiating hereditary hemochromatosis from secondary iron overload.

V. IRON AND FREE RADICAL FORMATION

Iron is essential for a wide variety of metabolic processes functioning primarily as an important biological catalyst (similar to many transition metals). Despite its physiologically beneficial role as a catalyst, iron can also have deleterious effects due to its ability to undergo changes in oxidation states involving one electron. The ability to access two oxidation states, ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), allows it to coordinate electron donors and to participate in redox processes. However, the characteristics that made iron an excellent catalyst also renders it potentially hazardous since redox reactions favor a reductive pathway which can lead to the formation of unstable intermediates with unpaired electrons, i.e., to "free radical" formation. The term "free radical" has been broadly defined by Halliwell and Gutteridge (84) as any species capable of independent existence that contains one or more unpaired electrons; an unpaired electron is one which occupies one atomic molecular orbital by itself. Acceptance of a single electron by the oxygen molecule, O_2 in its ground state (the most stable configuration of the O_2 molecule) results in the production of the superoxide radical, O_2^-. Addition of a second electron to the superoxide radical gives the peroxide ion, O_2^{2-}, which has no unpaired electrons; thus it is not a radical. At physiological pH the peroxide ion is immediately protonated to produce hydrogen peroxide, H_2O_2 and O_2; the reaction can be written:

$$2O^{2-} + 2H+ \longrightarrow H_2O_2 + O_2 \quad (1)$$

It should be noted that both the superoxide radical and hydrogen peroxide can be detected during normal metabolism in many biological

systems and there is sufficient evidence that they exist *in vivo* (85-92).

Due to the relatively weak O-O bond in H_2O_2, it is readily converted into two hydroxyl radicals (OH·); this reaction can be achieved by heat or by ionizing radiation. Additionally, a mixture of H_2O_2 and Fe^{2+} (ferrous iron) readily forms the OH· radical. This reaction is known as the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + Fe^{3+} + OH^{-} \quad (2)$$

Also, traces of Fe^{3+} (ferric iron) can react with H_2O_2 to produce the Fe^{2+} required in reaction (2):

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^{-} + 2H+ \quad (3)$$

Ferric iron also reacts with O_2^{-} to generate H_2O_2 and Fe^{2+}. This may result in the generation of the hydroxyl radical through a series of three reactions collectively known as the Haber-Weiss reaction:

$$O_2^{-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (4)$$

$$O_2^{-} + O_2^{-} \longrightarrow O_2 + H_2O_2 \quad (1)$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^{-} + Fe^{3+} \quad (2)$$

(The last step is Fenton's reaction).

Of the numerous reduction products derived from oxygen in the presence of iron, it is the hydroxyl radical, OH·, that is by far the most toxic. This free radical reacts with extremely high rate constants with most organic molecules found in cells. In particular, it attacks and destroys cell membranes, as well as DNA. In light of the damaging effects of the OH· radical, it is not surprising that aerobic organisms have evolved two enzymes to remove the two reactants, H_2O_2 and O_2^{-}, as rapidly as they appear to minimize the production of the OH· radical. The enzyme superoxide dismutase scavenges the O_2^{-} and glutathione

peroxidase (selenium enzyme) and catalase deal with H_2O_2. These enzymes are present in relatively large amounts in all aerobic organisms and their role, as well as the role of other molecules, in protecting biological systems against oxygen radicals is adequately documented (84). Since the toxicity of the O_2^- and H_2O_2 involves their conversion to the OH$^\bullet$ radical a reaction which in turn will depend on the amount of catalytic iron available, it can be seen that it is also important that iron be chelated in a controlled way *in vivo* so that uncontrolled free radical reactions are avoided.

In vertebrates most iron is tightly bound to proteins (i.e., transferrin, ferritin, lactoferrin) and would be unavailable for the Fenton reaction. The small pool of non-protein bound iron present could, however, conceivably provide iron for this reaction; physiological chelates, such as ferric citrate, have been shown to react with H_2O_2 *in vitro*. Normally this "free pool" is kept extremely small but "free iron" has been detected in individuals with iron-overload disease (93).

VI. NONENZYMIC LIPID PEROXIDATION

A free radical that has sufficient energy to abstract a hydrogen atom from a methylene carbon of a unsaturated fatty acid (LH) can initiate a chain reaction in bulk lipid. The resulting carbon-centered radical (L$^\bullet$) reacts rapidly with molecular oxygen to form a peroxy radical ($LO_2^\bullet$), which itself can abstract a hydrogen atom from an unsaturated fatty acid, leaving a carbon-centered radical and a lipid hydroperoxide (LOOH).

$LH + R^{\cdot} \longrightarrow L^{\cdot} + RH$ Initiation [1]

$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot}$

$LH + LO_2^{\cdot} \longrightarrow LOOH + L^{\cdot}$ Propagation [2]

$L^{\cdot} + L^{\cdot} \longrightarrow LL$

$LO_2^{\cdot} + LO_2^{\cdot} \longrightarrow LOOL + O_2$ Termination [3]

$LO_2^{\cdot} + L^{\cdot} \longrightarrow LOOL$

The free-radical chain reaction propagates (reaction 2) until two free radicals destroy each other to terminate the chain (reaction 3). In nonenzymic lipid peroxidation, the peroxy radicals last long enough to be able to move to new fatty acid molecules since they can readily be intercepted and scavenged by a variety of different antioxidants. Peroxidic products of the chain reaction (LOOH) are a complex mixture of isomers.

VII. FREE RADICALS, LIPID PEROXIDATION AND THE PATHOGENESIS OF IRON OVERLOAD DISEASE

In both primary and secondary iron overload conditions, ferritin, hemosiderin and transferrin become heavily loaded with iron. As previously mentioned, "free", non-protein bound iron in the form of low molecular weight complexes, has been found in the serum of some patients suffering from idiopathic hemochromatosis and transfusional siderosis (93,94). Although, iron accumulates in most organs of the body, severe clinical manifestations result from cytosiderosis of the liver and heart. The heart is particularly sensitive; in the past as many as one-third of untreated hemochromatotic patients died as a result of cardiac

failure or arrhythmia (12). In addition, many chronically-transfused thalassemics and juvenile hemochromatotics die during their second and third decades of life from congestive heart failure and cardiac arrhythmias (95). The ultrastructural appearance of iron-loaded cells from hemochromatotic individuals is characterized by an accumulation of electron-dense ferritin cores, mainly in the cytoplasm and by the presence of electron dense deposits of iron within the lysosomes (96,97). It has been hypothesized that these iron deposits are responsible for damaging lysosomal membranes, releasing hydrolytic enzymes into the cytoplasm, thus initiating cell damage (98,99). Work of Allison and Young (100) provided evidence that hydrolases released in vivo in cells can cause severe damage to cellular structure. Thus, the susceptibility of the lysosomal membrane to lipid peroxidation is of apparent importance to cellular pathology.

In a study of six patients with primary hemochromatosis and eight patients with secondary iron overload (all with thalassemia major), Peters and Seymour (98) demonstrated that lysosomes from liver biopsies of patients with iron overload were strikingly more fragile than control subjects. Also noted was that the lysosomal integrity of liver biopsies from patients with other types of cirrhosis were normal, thus indicating that the lysosomal changes were not due to a cirrhotic process affecting the homogenization procedure. Similar studies in biopsies from patients with different forms of chronic hepatitides including alcoholic liver disease have not demonstrated lysosomal abnormalities (101). Selden et al. (99) demonstrated a close positive correlation between enhanced lysosomal fragility and hemosiderin content of liver biopsies from patients with primary or secondary hemochromatosis. Thus, it was

suggested that the hemosiderin was responsible for the lysosomal disruption and subsequent tissue damage.

The mechanism of increased lysosomal fragility in hemochromatosis is uncertain. Early researchers proposed that distension of the lysosomes with poorly degradable ferritin and hemosiderin lead to rupture of their membranes (102). However, subsequent data show that lysosomal membrane rupture is not due to simple distension of the lysosomes (104) nor to an *in vitro* mechanical effect (103). Iron-generated free radicals have been implicated in lysosomal disruption by their ability to initiate a chain reaction of lipid peroxidation with lysosomal disruption *in vitro* (104-108).

VII. IRON AND IN VIVO LIPID PEROXIDATION

Numerous studies have documented evidence of enhanced lipid peroxidation in both naturally-occurring and experimental iron overload. Many of these studies demonstrated a protective role for various antioxidants, thus providing additional indirect evidence that lipid peroxidation is associated with iron overload *in vivo*.

Dougherty et al. (109) measuring exhaled ethane, a volatile autoxidation product of omega-3-unsaturated fatty acids demonstrated that vitamin E, the most important *in vivo* biological free radical scavenger, and selenium (a critical component of glutathione peroxidase, an enzyme which metabolizes hydrogen peroxide and lipid peroxides) protected rats against lipid peroxidation induced by acutely toxic doses of ferrous chloride and iron dextran. In a similar study, Dillard et al. (110) measured pentane (a volatile decomposition product of omega-6-unsaturated fatty acids) as an index of *in vivo* lipid peroxidation.

This study demonstrated that both dl-alpha-tocopherol (vitamin E) and L-ascorbic acid (vitamin C) effectively suppressed lipid peroxidation in rats injected intraperitoneally with iron dextran. Results suggested that vitamin E was much more effective than vitamin C as an antioxidant and N,N'-diphenyl-p-phenylenediamine was most effective among a group of synthetic antioxidants tested. These results confirmed previous findings (111,112). Bacon et al. (113) provided evidence of *in vivo* hepatic mitochondrial and microsomal lipid peroxidation as measured by conjugated diene formation in two models of experimental chronic iron overload in rats (i.e., parenteral ferric nitrilotriacetate and dietary supplementation with carbonyl iron). Results indicated that mitochondrial lipid peroxidation occurred at several hepatic iron concentrations in both models of iron overload; whereas, microsomal lipid peroxidation was detected only at the higher liver iron concentration achieved by dietary carbonyl iron supplementation. Thus, in light of these findings sufficient evidence is available to indicate that lipid peroxidation is associated with experimental iron overload *in vivo*.

Lipid peroxidation has also been demonstrated in naturally-occurring iron overload disease. Rachmilewitz et al. (114) showed that the erythrocytes of thalassemia major patients with transfusional iron overload underwent excess lipid peroxidation; this was associated with decreased levels of vitamin E. Heys and Dormandy (115) demonstrated that iron overloaded spleens from thalassemic patients were more susceptible to free radical peroxidation *in vitro*. In addition, it was found that tocopherol levels in these spleens correlated inversely with iron overload and homogenates with a low tocopherol content showed the

greater tendency to free radical oxidation, thus iron content was the main but not the only variable governing susceptibility to peroxidation. This study also investigated the effects of ascorbate on peroxidation and concluded that added ascorbate had a dose-dependent action either as an antioxidant or as a pro-oxidant, the direction of its effect depending mainly on the degree of iron overload.

IX. IRON AND MYOCARDIAL CELLS

Most of the studies of iron overload have been focused of the effect of excess iron on hepatocytes. However, iron cardiotoxicity remains the major life-limiting complication of thalassemia and other chronic anemias requiring continued transfusional therapy. Therefore, understanding the mechanism of myocardial iron toxicity is critical. Within the last decade, studies utilizing cultured neonatal rat myocardial cells have greatly enhanced the investigation of iron cardiotoxicity.

Early studies by Cox et al. (116) demonstrated that ferric ammonium citrate was readily taken up by cultured neonatal rat myocardial cells and that approximately 50% of the iron was sequestered in ferritin. Ultrastructural evaluation of these cells showed that endocytic vesicles and lysosomes contained iron-filled ferritin molecules. The number of lysosomes increased with time in both the control and experimental cultures; however the control cultures contained considerably fewer molecules of ferritin per lysosome than iron-treated cultures. Also, the number of ferritin molecules apparent in lysosomes increased with time and with increasing concentrations of iron in the medium.

Subsequent studies by Link et al. (117,118) correlated structural and biochemical alterations with functional derangement of myocyte contractility in cultured myocardial cells. Results of these studies indicated that roughly one-third of cellular radiolabelled iron was in ferritin and the rest in an insoluble lysosomal fraction (i. e., hemosiderin). However, iron uptake was almost completely inhibited by reducing the incubation temperature from 37°C to 10°C. Additionally, studies of iron-induced lipid peroxidation demonstrated that intracellular concentrations of malondialdehyde (MDA) were double after 15 minutes of iron loading and reached maximal concentrations at 3 hours; cellular MDA concentrations were normalized by iron mobilization using desferroxamine at concentrations ranging from 0.025 mmol/L to 0.3 mmol/L. Normalization of cellular MDA concentrations was in direct proportion to the amounts of iron removed. Functional studies determined the amplitude and rate of cell contractions using a Model 633 Video Analyser; results showed that exposure of myocardial cells to 20 μg/ml of iron gradually reduced the contractility and increased the rates and irregularity of the heart cell beats. In conclusion, these findings indicated that cultured myocardial cells were able to assimilate large amounts of nontransferrin iron and that iron uptake and mobilization were associated with striking changes in lipid peroxidation as manifested by the respective increase and decrease in cellular MDA concentrations. In addition, functional alterations were demonstrable at high concentrations of iron.

A recent study by Hershko et al. (119) investigated the ability of ascorbic acid, alpha-tocopherol and hypoxia to modify iron uptake, chelation, and toxicity as manifested by the generation of MDA in

myocardial cell cultures. Results indicated that ascorbic acid and tocopherol had opposing effects on iron uptake and cellular MDA production. Ascorbate inhibited iron uptake 73%, whereas tocopherol increased iron uptake by 19%. Contrastingly, the addition of ascorbate greatly enhanced the production of cellular MDA (86% relative to controls), however tocopherol reduced cellular MDA levels by 75%. Chelation studies (using desferroxamine) demonstrated significant reductions in cellular iron content (53%) and MDA production (40%). Neither simultaneous desferroxamine and ascorbate nor tocopherol treatment affected iron mobilization, however ascorbic acid entirely prevented the reduction of MDA concentration by desferroxamine in iron loaded cells. In contrast, tocopherol potentiated the antioxidant effect of desferroxamine.

The findings of Hershko's study indicating a marked increase in myocardial lipid peroxidation after ascorbate therapy underline the controversy regarding vitamin C supplementation in thalassemic patients (120). Ascorbate deficiency is a common complication of iron overload caused by the accelerated conversion of ascorbate to oxalic acid in the presence of excess tissue iron (121). Furthermore, ascorbate supplementation potentiates the *in vivo* effect of desferroxamine probably by enhancing storage iron mobilization and by increasing the chelatable iron pool (122). However, there have been reports of severe deterioration of cardiac function in primary and secondary iron overload patients receiving supplemental ascorbic acid to enhance responsiveness to desferroxamine therapy (123). Thus, experimental and clinical evidence indicates that ascorbic acid supplementation in iron overload

patients should be carried out with extreme caution, and in many cases may be entirely contraindicated.

Hershko's study was the first report to directly demonstrate a protective effect of alpha-tocopherol against lipid peroxidation in myocardial cells; however, the therapeutic use of alpha-tocopherol for protecting membranes against lipid peroxidation has been advocated in the past with evidence of limited success (124). In addition, this study as well as a previously mentioned study (118) indicated that desferroxamine was capable of reducing myocardial MDA concentrations in direct proportion to the amounts of iron removed. Thus, at least two methods of protecting myocardial cells from increased lipid peroxidation are available: removal of excess iron by a metal chelator or preventing the propagation of lipid peroxidation by the chain-breaking antioxidant alpha-tocopherol.

X. IRON AND INFECTION

Iron is essential for the growth of virtually all bacterial organisms (125-127). Weinberg (125) demonstrated that the concentration of iron necessary to promote bacterial growth ranged from 0.4 to 4.0 uM; this quantity of iron is present in many environments, including mammalian tissues. However, nearly all of the iron in mammals is complexed to intracellular proteins (i.e., ferritin, hemosiderin, heme) or tightly bound extracellularly to high affinity iron-binding glycoproteins, such as transferrin in serum and lymph; as well as a related protein, lactoferrin in external secretions and milk (128-130). Although there is a wealth of iron in mammalian tissues and fluids, the amount of free iron in equilibrium with iron-binding proteins was

calculated by Bullens et al. (131) to be approximately 10^{-18}M, which is virtually zero. This result was later confirmed by other researchers (129,130). Thus, under normal conditions the amount of free iron available to sustain bacterial growth is much too small (131,132).

A. Siderophores

Bacteria are known to possess two iron transport systems, a low affinity system that functions when iron is freely available and a high affinity system which operates during periods of iron restriction. A critical component of the high affinity system is the synthesis and release of low molecular weight iron chelators, known as siderophores (126,133). Siderophores bind and solubilize ferric iron, re-enter the bacterial cell (via special outer membrane proteins in many cases) where the iron is utilized to promote microbial growth. These iron chelators are categorized into two broad chemical classes, hydroxymates and catecholamides; at least one of which has been identified in almost every aerobic or facultatively anaerobic bacterial species examined (126).

The hydroxymate class of siderophores is typified by desferrioxamine B, which possesses an iron ligand formation constant of 10^{31} M^{-1} (134). Desferrioxamine B is synthesized by *Streptomyces pilosus* and is used clinically as a metal chelator to treat iron and in some cases aluminum overload (94,135,136). Numerous reports have documented the promotion of *Yersinia enterocolitica* septicemia by desferrioxamine in patients with both acute and chronic iron overload (137-140). Similar case reports demonstrated an increased incidence of disseminated mucormycosis in desferrioxamine treated patients with

aluminum overload resulting from long-term dialysis (141-144). *Y. enterocolitica* does not produce siderophores but has receptors for the iron-siderophore complex (145). It is known that desferrioxamine acts as a growth factor for *Y. enterocolitica in vitro* and dramatically enhances the virulence of this organism in experimental infections by providing iron that can be utilized for microbial growth (138,146-148). A similar mechanism is thought to operate with respect to the development of mucormycosis (142,144).

Alternatively, other reports have suggested that treatment with desferrioxamine does not favor the development of septicemia or bacterial infection independently of iron overload and that the iron load of the host is the most important predisposing factor leading to infection (149).

The catecholamides, represented by enterochelin (also called enterobactin), generally form tighter iron complexes than hydroxymate siderophores. Enterochelin is synthesized by several genera of bacteria (e.g., *Klebsiella, Escherichia, Salmonella, Shigella*) and has the highest formation constant for ferric iron ever recorded among chelators, being near 10^{52} M^{-1} at neutral pH (134,150-155). Thus, organisms synthesizing enterochelin are capable of competing for complexed iron (e.g., transferrin - Kf approx. 10^{28} M^{-1}) due to the large difference in formation constants.

Pseudomonas species are known to produce several siderophores: pyochelin, pyoverdin, pseudobactin, ferribactin and ferrioxamines (156-161). However, only pyochelin and pyoverdin are of significant interest with regards to infection. Pyoverdin has been demonstrated to promote the growth of *Pseudomonas* in the presence of transferrin (162), despite

having a binding constant for iron of only 10^{32} (163). Cox (164) demonstrated that pyochelin has dramatic effects on the virulence of *Ps. aeruginosa* and promotes experimental infections in mice. Additionally, work by Ankenbauer et al. (165) suggest that the production of pyoverdin is critical for the serologic growth of *Ps. aeruginosa*; apparently pyochelin is poorly synthesized by the organism in serum and offers minimal benefit.

XI. THE IRON-WITHHOLDING SYSTEM

The iron withholding system in mammalian species is composed of both storage (e.g., ferritin) and transport (e.g., transferrin, lactoferrin) proteins. Of the several iron containing proteins it is known that the transferrins have well-defined bacteriostatic properties.

A. Transferrins

Schade and Caroline (166) observed that raw egg white was inhibitory to bacterial growth and that such inhibition could be abolished by the addition of iron. The protein responsible for this phenomenon was identified as conalbumin (later referred to as ovotransferrin by Alderton et al.) (167) and they noted that it prevented microbial growth by withholding iron from bacterial and fungal invaders; it was proposed that a related protein was present in the plasma.

In 1946, Schade and Caroline (168) identified an iron-binding plasma protein and applied the name siderophilin. One year later, Holmberg and Laurell (169) changed the name to <u>transferrin</u> because they discovered that the protein had a second function of transporting iron among the various tissues of the body.

The various members of the transferrins are glycoproteins (each consist of a single chain of approx. 680 amino acids) with molecular weights in the range of 75,000-80,000. They contain two metal binding sites (each composed of one aspartyl, one histidinyl, and two tyrosyl residues) in the polypeptide and are capable of binding up to two atoms of ferric iron at neutral pH values. This binding of iron is accompanied by the binding of a carbonate ion on a 1:1 molar basis (170).

The primary function of plasma transferrin is the transport of iron to and among cells of erythropoietic bone marrow, spleen, liver, small intestine, muscle, as well as the reticuloendothelial system (171). However, considerable evidence suggest an addition microbiostatic function. Kochan et al (172) clearly showed that the amount of available iron (i. e., the ratio between iron saturated and iron-free transferrin) determined the ability of tubercle bacilli to grow in serum samples. A low value for the ratio (e. g., human serum, 0.4), was associated with tuberculostasis, whereas a high value (e. g., guinea pig serum, 5.6) was affiliated with support of bacillary multiplication. Additionally, the degree of bacillary growth correlated with the level of transferrin saturation in various mammalian species. Human and bovine sera with a transferrin saturation level of approximately 30% were tuberculostatic, whereas guinea pig serum (Tf saturation = 84.4%) sustained bacillary growth. Limited bacillary multiplication was observed in rabbit and mouse sera (Tf saturation approximately 60%).

Lactoferrin was discovered in milk in 1939 but not definitively identified until 1960 (173). In human milk it comprises 20% of the

protein content (129), however this figure is reduced to only 2% in bovine milk. In addition, lactoferrin is an important component of other exocrine fluids such as bronchial mucous, nasal exudate, saliva, gastrointestinal fluid, bile, seminal fluid and tears.

Bezkorovainy (174) suggested that the relatively large quantity of lactoferrin in human milk is an important contributor to the much lower incidence of infection in breast-fed infants as compared with the incidence in infants receiving milk formula or bovine milk. In addition to its ability to retain iron from potential infant intestinal pathogens such as *Escherichia*, *Salmonella*, and *Clostridium*, the lactoferrin in human milk may function to retard the absorption of intestinal iron (derived from both bile and diet) in the human nursling (175). This inhibition of iron absorption is significant because as previously stated the level of saturation of Tf is believed to be critical in the development of infection. Values (of Tf saturation) less than roughly 30% are helpful in preventing infection (176).

Lactoferrin differs from transferrin and ovotransferrin in that it retains its binding avidity for ferric iron far below neutrality (pH < 4.0). This ability makes it an effective scavenger of iron in septic areas in which the pH has been decreased by organic acids derived from microbial invaders, as well as from infiltrating inflammatory cells, namely polymorphonuclear leukocytes (PMNs) of which lactoferrin is a major component (177). Upon migration of PMNs to a site of sepsis degranulation of specific granules occurs causing the release of lactoferrin. In the septic area the unsaturated lactoferrin combines with the increased iron pool that is derived from dying microorganisms and injured tissue cells. The binding of free iron by lactoferrin

serves to retain iron from utilization by microbial pathogens. In addition, the saturated lactoferrin is ingested by macrophages which provides a signal to suppress continued metabolic activity. This signal aids in the down-regulation of the inflammatory process that occurs at the conclusion of the successful response to insult (178). It should be noted that humans with PMNs deficient in specific granules containing lactoferrin are at increased risk of gram-positive and gram-negative bacterial infections (179).

XII. IRON AND CELLULAR IMMUNITY

A. NK Cells

Numerous studies investigating the correlations between iron status and immune function in clinical situations have been conducted in thalassemic patients. Akbar et al. (180) noted a reversible, transfusion-related decrease in natural killer (NK) cell function in B-thalassemia major patients who were iron overloaded as a result of chronic transfusion therapy. The NK cell function was significantly increased when effector cells were preincubated with an iron chelator (desferrioxamine or 2,3-dihydroxybenzoic acid), thus indicating that the decrease was related to iron overload. (Note: Similar results were not observed when target cells were incubated with chelators). It was suggested that iron may influence NK activity by causing changes in the expression of transferrin receptors. Transferrin receptors (TfR) have been proposed to be a target structure for NK cells (181,182) and that TfR on both effector and target cells are important in the recognition event of cell killing (183). Mattia et al. (184) suggested that the expression of this receptor can be manipulated by agents (such as

desferrioxamine) that alter the iron status of cells. Thus, it was proposed that the decreased NK cell activity seen in thalassemia patients is the result of decreased TfR expression due to iron overload. In addition, the observation that preincubation of thalassemic effector cells with iron-chelating agents can increase NK function may be related to an increase in the expression of TfR due to removal of iron. Alternatively, work by Shau et al. (185) proposes that the TfR has an important role in the induction of NK-like activity in long-term culture with IL-2 and argued against its role as a target recognition determinant. They suggest that the importance of the TfR is likely related to its direct interaction with Tf during the induction phase of NK-like function rather than in its unspecified role during effector phase cytotoxicity.

B. Polymorphonuclear granulocytes (PMN)

There have been several reports relating the influence of iron on the phagocytic function of polymorphonuclear granulocytes (PMN). *In vitro* the phagocytic ability of PMN were demonstrated to be reduced after incubation with ferrous, as well as ferric iron (186-190). Similarly, PMN from patients with iron overload have also exhibited impaired phagocytic function (191-193). This impairment is thought to mediated by non-transferrin plasma iron found in primary and secondary iron overload patients (93,94,194,195).

Waterlot et al. (192) demonstrated altered neutrophil phagocytosis (of *Staphylococcus aureus*) and myeloperoxidase activity in a group of polytransfused patients receiving maintenance hemodialysis. Results indicated that the index of phagocytosis was inversely correlated with

serum ferritin concentrations and following 6 to 18 weeks of desferrioxamine therapy phagocytic function and myeloperoxidase activity returned to normal in most patients. These results confirmed *in vitro* studies (196) in which neutrophil phagocytosis was altered in an iron enriched medium and corrected by the addition of desferrioxamine.

Cantinieaux et al. (193) documented both altered PMN phagocytosis and killing of *Y. enterocolitica* in a similar group of hemodialysis patients. Results indicated that the reduced phagocytosis may be the consequence of a cellular defect since opsonization with normal serum did not improve phagocytic function. A previous study in thalassemia major patients (197) revealed a similar defect in PMN phagocytosis of E. coli. Prussian Blue positive staining of blood PMN indicated a cellular iron intoxication to explain phagocytic dysfunction. Excessive production of toxic oxygen species under the influence of an increase in cellular iron is thought to be involved in the pathogenesis of impaired phagocytosis. These radicals have been reported to be toxic for cellular membranes (198,199).

Reduced phagocytosis and killing are not the sole alterations in PMN function associated with iron overload. Khan et al. (200) demonstrated defective chemotactic and random migration patterns in a group of 11 thalassemia major patients. It was not determined if this reflected a primary defect of PMN or was a secondary manifestation of associated liver disease and/or diabetes mellitus.

C. Peripheral Blood Monocytes (PBMo)

Ballart et al. (201) demonstrated that PBMo from thalassemia major patients exhibited markedly diminished lytic activity against C.

pseudotropicalis, however phagocytic activity did not vary significantly from controls. In addition, an inverse correlation was documented between PBMo lytic activity and serum ferritin levels. The results from this study suggested that PBMo from patients with thalassemia major have an intracellular defect in their microbicidal mechanisms associated with the degree of iron overload.

D. Lymphocytes

Studies examining the relationship of iron overload, lymphoid subsets and immune function have focused on patients with thalassemia intermedia (202-204) and major (180,205,206). Lymphocytes from a group of thalassemia intermedia patients exhibited diminished mitogen responses to stimulation with PHA and Con A but not PWM (203). In polytransfused thalassemia major patients, Grady et al. (205) observed increasing proportions of circulation CD8+ cells that correlated significantly with the number of transfusions.

In IHC patients two conditions appear to persist: (1) unusually high numbers of thermostable E-rosette forming cells (207), and (2) Bjorn-Rasmussen et al (208) demonstrated a high percentage of PBMo expressing receptors for transferrin in many patients.

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CHAPTER I

GRAM NEGATIVE SEPSIS IN IRON LOADED GUINEA PIGS

Abstract

Susceptibility to infection is a well recognized manifestation of human iron overload disorders. Existing animal models of iron overload are time consuming, expensive, and have not been developed for the study of severe spontaneous gram negative infections. Experiments with the guinea pig model of iron overload demonstrated that animals receiving relatively large intraperitoneal injections of iron dextran (200 mg/kg every other day for five injections) have a high incidence of spontaneous gram negative infections. *Pseudomonas* was isolated from the liver, spleen or blood of 71% of the iron treated guinea pigs (n=7). At necropsy the affected animals had markedly icteric mucous membranes, roughened hair coats, and numerous small multifocal regions of hepatic necrosis (6/7). Histologically, these randomly distributed areas of coagulative necrosis were variably-sized with few inflammatory cells. Control animals (n=4) appeared clinically normal and without gross or histologic lesions. We conclude that guinea pigs receiving relatively large injections of iron dextran have a high incidence of gram negative infections and will provide a useful model for further investigation of iron induced sepsis.

I. INTRODUCTION

Acute and chronic iron overload is associated with an increased susceptibility to infection (1-5). Treatment of both iron and aluminum overload patients with desferrioxamine (Df), a metal chelator, mobilizes metal stores and enhances the probability of life-threatening infection (2-6).

Several mechanisms may be involved in the development of enhanced susceptibility to infection in iron overload patients: [1] saturation of the host iron withholding system resulting in diminished bacteriostatic effectiveness of iron-binding glycoproteins, transferrin and lactoferrin (7,8); [2] increased virulence of bacterial pathogens in the presence of freely available iron (9); [3] altered cellular immune responses involving several types of leukocytes (10-16).

We report an increased incidence of spontaneous gram negative infections using guinea pigs treated for less than two weeks with relatively large doses of intraperitoneal iron dextran.

II. METHODS

Seven female Hartley guinea pigs (Michigan Dept. of Public Health) weighing 200-250 grams received 5 intraperitoneal injections of 200 mg Fe/kg of iron dextran (Sigma Chemical Co., St. Louis, Mo) every other day. Control animals (n=4) were injected with equivalent volumes of dextran. Animals were humanely sacrificed and tissues collected for microbiologic and histopathologic evaluation. Samples of liver, spleen and blood were cultured from iron treated animals. Specimens were cultured separately on both blood agar and McConkey's agar. Tissues

were fixed in 10% buffered formalin and stained with hematoxylin and eosin for light microscopy; gram staining was performed in select cases.

III. RESULTS

Clinically, all iron treated guinea pigs were markedly jaundiced with evidence of reduced weight gain and roughened hair coats; the control group appeared healthy and only liver samples were submitted for evaluation.

Pseudomonas aeruginosa was cultured from the blood, liver or spleen of iron dextran treated (5/7; 71.4%) guinea pigs (Table 1). *Pseudomonas* was also cultured from the liver of a single control guinea pig (1/4; 25%), however this was believed to be a subclinical infection as there were no clinical signs nor pathologic lesions in this animal.

Macroscopically, the liver from 6 of 7 of the iron treated animals was discolored (brownish-yellow) and contained numerous, multifocal (<0.5 mm) regions of necrosis distributed throughout the parenchyma. In addition, moderately-sized (1-2 cm) subcutaneous abscesses were present at 4 of 7 sites of injection; similar lesions were not observed in the control group. Histologically, randomly dispersed variably-sized, multifocal regions of hepatic coagulative necrosis were observed in iron loaded guinea pigs (Figure 1); inflammation was minimal in these areas. Severe fibrinous peritonitis associated with numerous gram-negative bacterial organisms developed in one of seven iron treated guinea pigs (Figure 2). Other histologic lesions in this animal included chronic, active fibrosing septal panniculitis and myositis in subcutaneous tissues. These lesions were morphologically consistent with resolving

TABLE 1: Culture Results

Group	Liver	Spleen	Whole Blood
Iron Dextran 1.0 g Fe/kg (N = 7)	Ps. aeruginosa	Ps. aeruginosa	Ps. aeruginosa
	Ps. aeruginosa	Negative	Negative
	Negative	Ps. aeruginosa	Negative
	E. coli	Ps. aeruginosa	Ps. aeruginosa
	Negative	Negative	Negative
	Negative	Negative	NT
	Ps. aeruginosa S. odorifera	Ps. aeruginosa S. odorifera	NT
Dextran Control (N = 4)	Ps. aeruginosa*	NT	NT
	Negative	NT	NT
	Negative	NT	NT
	Negative	NT	NT

NT = Not Tested * Subclinical

Injected iron dextran was negative for bacterial growth.

Figure 1. Focal region of hepatic necrosis with pyknotic nuclei (arrows) and extracellular iron particles bordered by nondegenerate cells. H & E; Line = 100μm

Figure 2. Numerous rod-shaped bacterial organisms (arrows) surrounded by heterophils and macrophages in the liver of a septic guinea pig. H & E; Line = 20 μm.

Figure 1

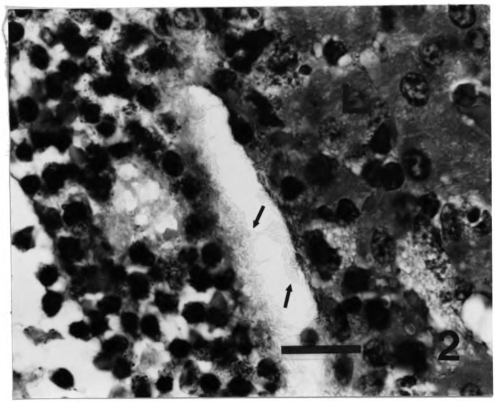
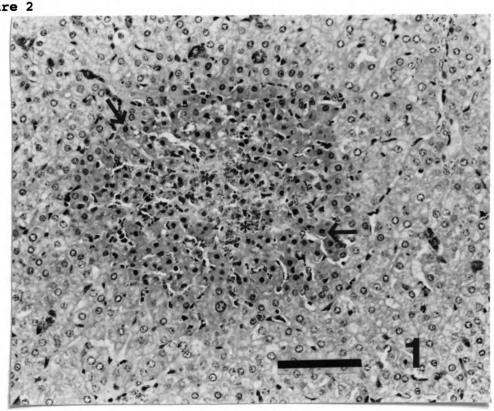


Figure 2



abscesses or cellulitis. A single sample of this tissue cultured positive for both *Pseudomonas aeruginosa* and *Serratia odorifera*.

IV. DISCUSSION

This report documents an increased occurrence (71%) of *Pseudomonas* infection in guinea pigs given high levels of intraperitoneal iron dextran. These findings support prior studies in humans and animals relating iron to infection in both clinical, as well as experimental studies using mice, rats and guinea pigs (17-19). The median lethal dose of intraperitoneally administered *Yersinia enterocolitica* was reduced 10 to 100-fold in mice injected with iron dextran; parallel results were demonstrated using guinea pigs in a keratoconjunctivitis test (17,18). In addition, iron has been demonstrated to enhance the virulence of *Pseudomonas aeruginosa* in a chronic pulmonary infection model in rats (20) and enhanced the growth of *Staphylococcus aureus* and *Yersinia enterocolitica* in human serum (19). The virulence of *Listeria monocytogenes, Klebsiella pneumoniae* and *Escherichia coli* have also been shown to be accentuated by the injection of iron (9).

Numerous reports relate iron to infection in a variety of clinical circumstances. Parenteral administration of iron dextran to iron deficient Polynesian infants was temporally related to an outbreak of *E. coli* neonatal sepsis (1). In addition, an unusual cluster of *Acinetobacter pneumonia* was reported in foundry workers inhaling high concentrations of iron particles (21). Several studies document an enhanced incidence of *Yersinia enterocolitica* septicemia in both acute and chronic iron overload patients, particularly those treated with desferrioxamine (2-4). The role of desferrioxamine is debated, however

it should be noted that recent studies describe an increased occurrence of mucormycosis in desferrioxamine-treated renal dialysis patients with aluminum overload (5,6).

The development of enhanced bacterial virulence observed with iron overload may involve inhibition of transferrin and lactoferrin, whose bacteriostatic capability is known to be inversely related to their level of saturation (7). The level of Tf saturation in this report was not determined. However, prior experiments from this laboratory demonstrated complete saturation of transferrin (>100%) in guinea pigs receiving 25% (250 mg/kg) of the amount of injected iron dextran in this report. Normal guinea pig Tf saturation is 85% thus, the amount of injected iron required to thoroughly saturate iron-binding proteins was relatively small.

Iron overload alters other immune defense systems. Studies in iron overload patients have shown depressed cellular immunity as evidenced by decreased natural killer (NK) cell activity (10), suppression of polymorphonuclear granulocytes (PMN) phagocytic function (13-16), reduced lytic activity in peripheral blood monocytes and diminished mitogen responses in lymphocytes (22-24). It remains unknown as to how these and possibly other iron induced defects interact to produce sepsis.

Results in this report provide additional evidence that iron is capable of enhancing the development of sepsis in the mammalian host. We propose that the iron dextran injected guinea pig mimics iron-induced septicemia observed in human patients and will provide a model to investigate the relationship between iron overload states, host immunity and bacterial virulence. In addition, pertinent related questions

involving the efficacy of prophylactic or therapeutic antibiotics, as well as chelation treatment could be examined using this model.

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CHAPTER II

IRON INDUCED MYOCARDIAL AND HEPATIC LYSOSOMAL ABNORMALITIES IN THE GUINEA PIG.

I. INTRODUCTION

Primary and secondary iron overload disorders are characterized by the development of cytosiderosis with accumulation of iron in lysosomes of both reticuloendothelial and parenchymal cells of the liver, spleen, heart, pancreas and other tissues. As a consequence of severe siderosis a variety of clinical disorders may evolve including hepatic cirrhosis, cardiomyopathy, endocrine pancreatic dysfunction, bronze skin pigmentation and hypogonadism.

The mechanisms of tissue damage in iron overload diseases are not clearly defined, however previous research suggests that iron-mediated lysosomal disruption may play a role in initiating cell injury. Numerous reports demonstrate that hepatic lysosomes from iron overload patients are particularly fragile when compared to control subjects (1-4). Similar findings are documented in rats fed carbonyl iron (5,6), injected with an iron-sorbitol-citric acid complex (7) or iron nitrilotriacetate (8). Favored, mutually compatible, proposals for the mechanism of lysosomal membrane injury in iron overload include (a) accumulation of excess hemosiderin leading to physical disruption of the lysosome and (b) iron-catalyzed lipid peroxidation mediating the loss of lysosomal membrane integrity. Following the lysosomal membrane injury,

it is hypothesized that leakage of acid hydrolases results in destruction of intracellular constituents eventuating in cell damage (9).

Severe cardiomyopathy is a common clinical manifestation of both hereditary and transfusion-induced iron overload. Chronically transfused thalassemic children with iron cardiomyopathy may develop severe clinical problems or even die during puberty. Investigation of this problem has been hampered by lack of an easily reproducible animal model that mirrors the human condition. Although iron overload has been reproduced in rats by injection of iron-sorbitol-citric acid complex or feeding carbonyl iron, these models are expensive and require 3 weeks-12 months before the animals are iron loaded and studies can begin (5,10,11).

We have developed a previously unexplored animal model of iron overload. Guinea pigs intraperitoneally injected with iron dextran develop hemosiderosis of both their livers and hearts. Iron loaded animals demonstrate increased serum iron, total iron binding capacity, and percent transferrin saturation. This model was used to investigate the pathophysiology of iron induced toxicity; abnormalities involving membrane peroxidation and enhanced liver and myocardial lysosomal enzyme release are presented.

II. MATERIALS AND METHODS

Animal Treatment

Female Hartley guinea pigs (Michigan Department of Public Health) weighing 200-250 grams were housed (4-6/group) in stainless steel cages on wire grates and fed a commercial diet with vitamin C (Purina Guinea

Pig Chow # 5025, St. Louis, MO). Iron dextran (Sigma Co., St. Louis, MO) [83 mg Fe/kg/injection (pH 7.4)] was injected intraperitoneally on alternate days to achieve iron loading levels of 0.25, 0.5, 1.0 and 2.0 g Fe/kg BW for lysosomal studies and 0.5 and 1.5 g Fe/kg BW for the malondialdehyde study. Controls received an equivalent volume of dextran. All animals were sacrificed 48 hours following the final injection.

Sample Preparation

Blood was collected via aortic puncture from guinea pigs anesthetized with pentobarbital in combination with ether. Blood was flushed from liver vasculature with 50 ml of ice-cold .25 M sucrose with 1 mM disodium EDTA and 10mM Hepes (pH 7.4). The right lobe of the liver was immediately excised and placed in the perfusion solution. Excised hearts from the same guinea pigs were placed in an ice-cold .25 M KCl and 1 mM disodium EDTA with 10 mM Hepes (pH 7.4). Tissues were blotted, weighed and minced with scissors; livers placed in 10% (w/v) sucrose and hearts in isotonic KCL. All subsequent steps were conducted at 0-4°C. Livers were homogenized for 45 seconds (s) with a Potter-Elvehejm homogenizer with a Teflon pestle. Hearts were homogenized using a Polytron Kinematica homogenizer (Brinkmann Instruments) for 5s at a low speed followed by a 30s homogenization with a Potter-Elvehejm homogenizer

Serum Iron, TIBC and Tf Saturation

Serum iron and total iron binding capacity were determined colorimetrically using ferrous ammonium sulfate as a standard (Stanbio Laboratory, Inc., San Antonio, TX). This method was a modification of

that reported by Persijin et al., (12) employing the chromogenic compound, ferrozine, as described by Stookey (13).

Lysosomal Enzyme Assays

Fractions

Following centrifugation of the whole homogenates at 800 x g for 10 minutes the enzyme activity of the supernatant fraction in the presence of 0.25 (w/v) Triton X-100 was termed the total enzyme activity. Free activity indicates that measured in the 22,000 x g (30 minutes) supernatant fraction in the presence of 0.2% (w/v) Triton X-100. The free activity is expressed as a percentage of the total activity without regard to quantity of protein.

Reactions were performed at three enzyme concentrations to verify linearity; all assays were demonstrated to be linear with time. Acid phosphatase (EC 3.1.3.2) was measured by a modification of the method of Vaes and Jacques (14); the pH was 5.0 as described by Schroeder et al. (15). Serum and tissue N-acetyl-B,D-glucosaminidase (EC 3.2.1.30) was assayed according to Sellinger et al. (16) using p-nitrophenyl-N-acetyl-B-D-glucosaminide as substrate. N-acetyl-B,D-glucuronidase (EC 3.2.1.31) was assayed according to Schroeder et. al (15). with the modification that both free and total activities were incubated for 60 minutes. Serum protein was estimated according to Lowry et al. (17) with bovine serum albumin as the standard.

Malondialdehyde Determination

Malondialdehyde in whole homogenates of liver and heart was estimated using thiobarbituric acid as described by Ohkawa et al. (18).

The protocol were slightly modified by centrifuging tubes at 175 x g for 20 min. to form the organic layer (pink chromogen).

Statistical Analysis

All data was analyzed using an unpaired t-test. Percentage values were normalized by arcsin square root percentage transformation (19).

II. RESULTS

Serum Iron, Serum TIBC and Transferrin Saturation

Significant elevations in serum iron values occurred at total iron doses of 0.5 ($p<0.001$) and 1.0 g/kg ($p<0.05$); serum TIBC values were also increased at these dose levels ($p<0.001$ and 0.01, respectively). Transferrin saturation was elevated at both 0.25 and 1.0 g Fe/kg BW ($p<0.01$) (Table 2).

Hepatic Lysosomal Fragility

Iron loaded animals at all dose levels had increased lysosomal fragility as demonstrated by a significant elevation in the free activity of hepatic glucosaminidase ($p<0.01$) and B-glucuronidase ($p<0.05$) (Figures 3 and 4). Elevation of free glucosaminidase and glucuronidase occurred at the lowest iron dose level (0.25 g/kg) and did not differ significantly between groups of iron treated animals, indicating an initial maximal or near maximal release of these hydrolases with no sequential increase at higher iron loads. Free hepatic acid phosphatase activity was significantly elevated at 0.5, 1.0 and 2.0 g Fe/kg BW ($p<0.01$) (Figure 5).

Table 2: Comparison of Serum Iron, Total Iron Binding Capacity (TIBC), and Transferrin Saturation Values in Dextran Control (Con) versus Iron Treated (Fe) Guinea Pigs.

Dosage (g/kg)			Serum Iron (ug/dl)	TIBC (ug/dl)	Transferrin Saturation (percent)
0.25	Con	(n=4)	245.80± 34.50	274.75± 27.72	89.70±13.07
	Fe	(n=6)	266.50± 26.60	242.16± 18.66	°109.84± 3.90
0.50	Con	(n=6)	267.16± 25.94	273.50± 25.94	97.81± 5.92
	Fe	(n=6)	■837.50±253.32	■953.83±248.54	87.41± 8.54
1.0	Con	(n=6)	295.00± 27.62	310.40± 29.05★	97.55± 4.96★
	Fe	(n=5)	•473.75±135.64	°450.66± 76.84★	°115.84± 5.27★

Normal Guinea Pig Values
(Kochan, et. al, J Bact, 100:64:70)
TIBC = 323 ug/dl
Serum Iron = 273 ug/dl
Transferrin Saturation = 84.4%

• = $p<0.050$
° = $p<0.010$
■ = $p<0.001$
★ (n=5)
☆ (n=3)

Figure 3:

Comparison of Free Hepatic B-N-acetylglucosaminidase (NAG) Activity.

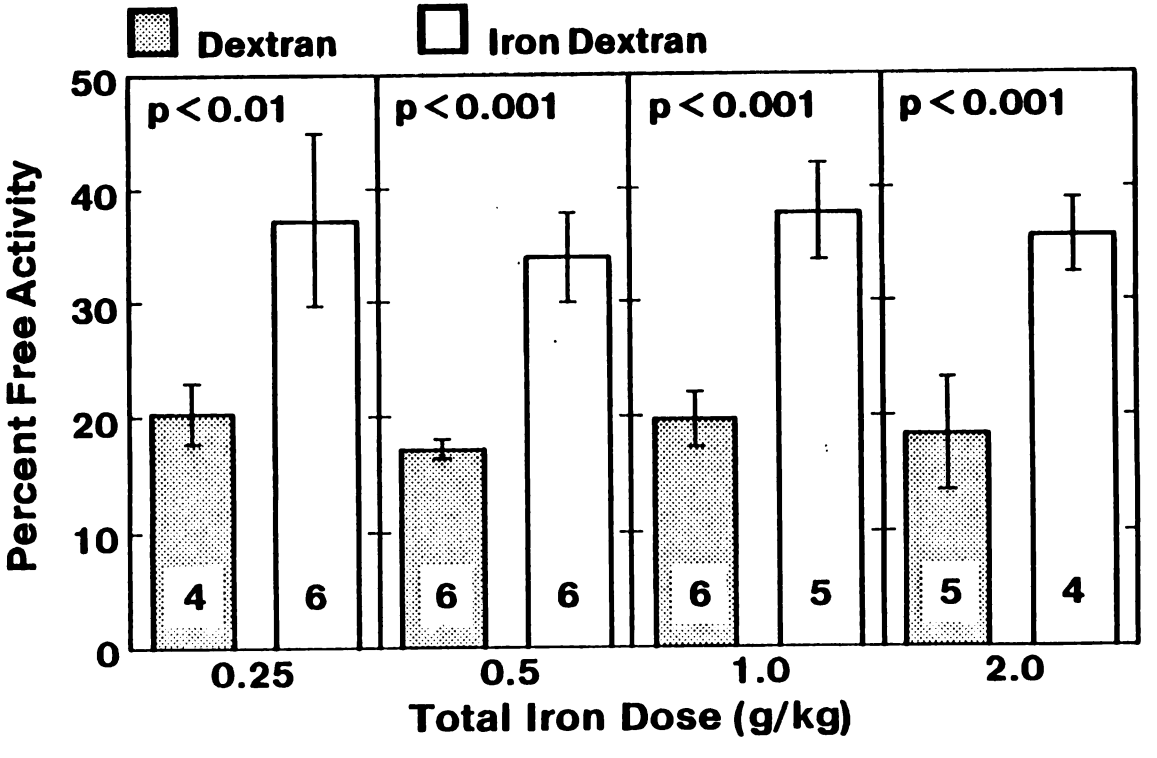

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Figure 4:

Comparison of Free Hepatic B-glucuronidase in Dextran Control and Iron Treated Guinea Pigs.

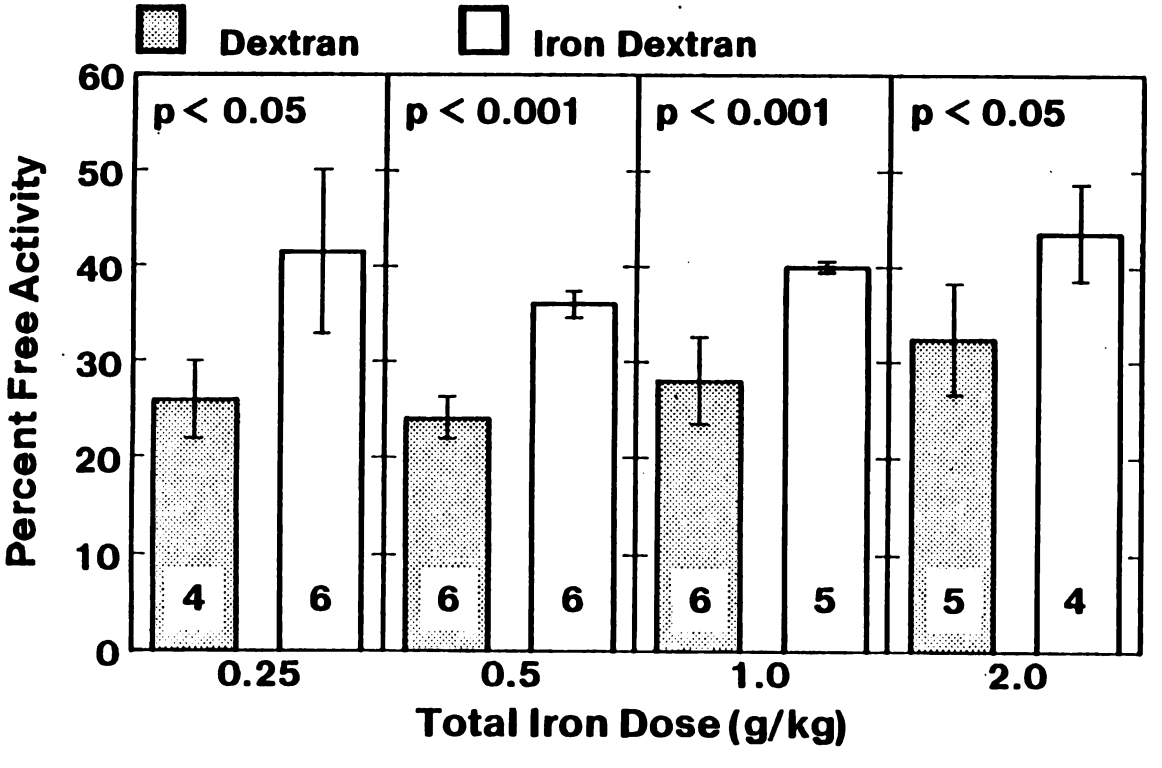

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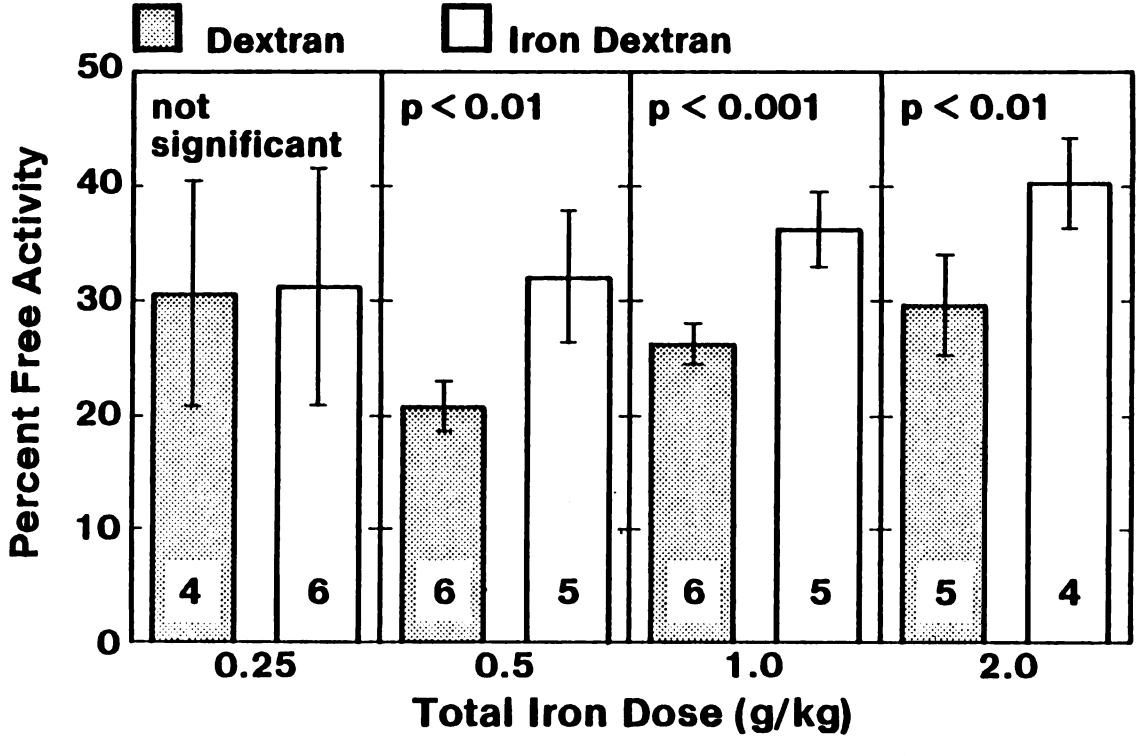
Figure 5:

Comparison of Free Hepatic Acid Phosphatase in Dextran Control and Iron Dextran Treated Guinea Pigs.



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Serum Glucosaminidase

Levels of serum glucosaminidase activity were measured in an effort to relate iron toxicity to changes in serum enzyme activity. Similar to the findings with hepatic glucosaminidase there was a significant elevation of serum glucosaminidase activity (Figure 6) at all levels of iron loading ($p<0.01$); at 0.25, 0.5, and 2.0 g Fe/kg the increase was approximately 2-fold over control values and did not vary significantly between iron treated groups.

Myocardial Lysosomal Fragility

The pattern of lysosomal enzyme abnormalities differed from that observed in the liver. Free myocardial glucosamindase (Figure 7) was significantly elevated at all iron dose levels ($p<0.05$). However, the free activity of myocardial acid phosphatase (Figure 8) was increased only at the highest iron dose of 2.0 g Fe/kg ($p<0.05$).

Generation of Malondialdehyde in Whole Homogenates of Heart and Liver

Levels of malondialdehyde (Figure 9) were significantly elevated only at the high dose (1.5 g Fe/kg BW) of iron loading in whole homogenates of both heart and liver ($p<0.01$).

IV. DISCUSSION

Investigation of the pathophysiology of excess iron toxicity utilizes the guinea pig model which is less expensive and requires a shortened iron loading time than previously developed animal models (5,10,11). Like humans with iron overload, guinea pigs injected with iron have increased serum iron and percent transferrin saturation. Although previous studies have investigated the fragility of hepatic

Figure 6:

Comparison of Serum B-N-acetylglucosaminidase Activity in Dextran Control and Iron Dextran Treated Guinea Pigs.

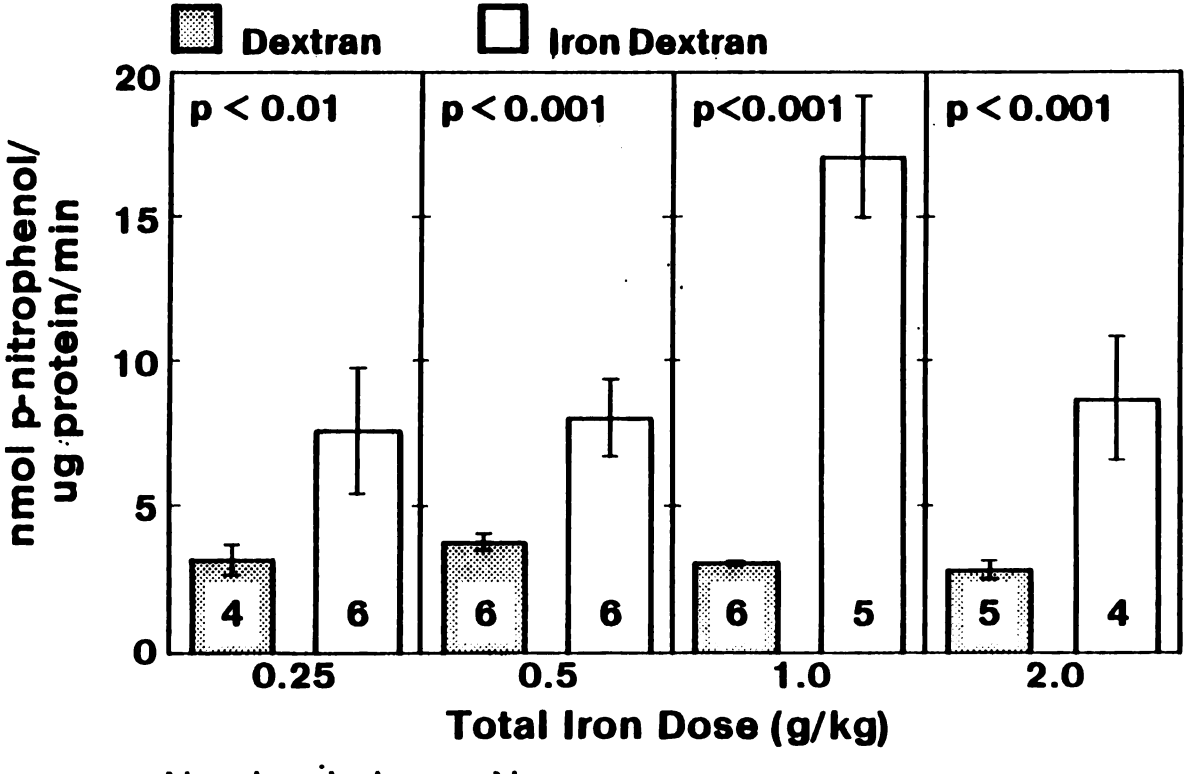

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Figure 7:

Comparison of Free Myocardial B-N-acetylglucosaminidase in Pooled Dextran Controls vs. Iron Treated Guinea Pigs.

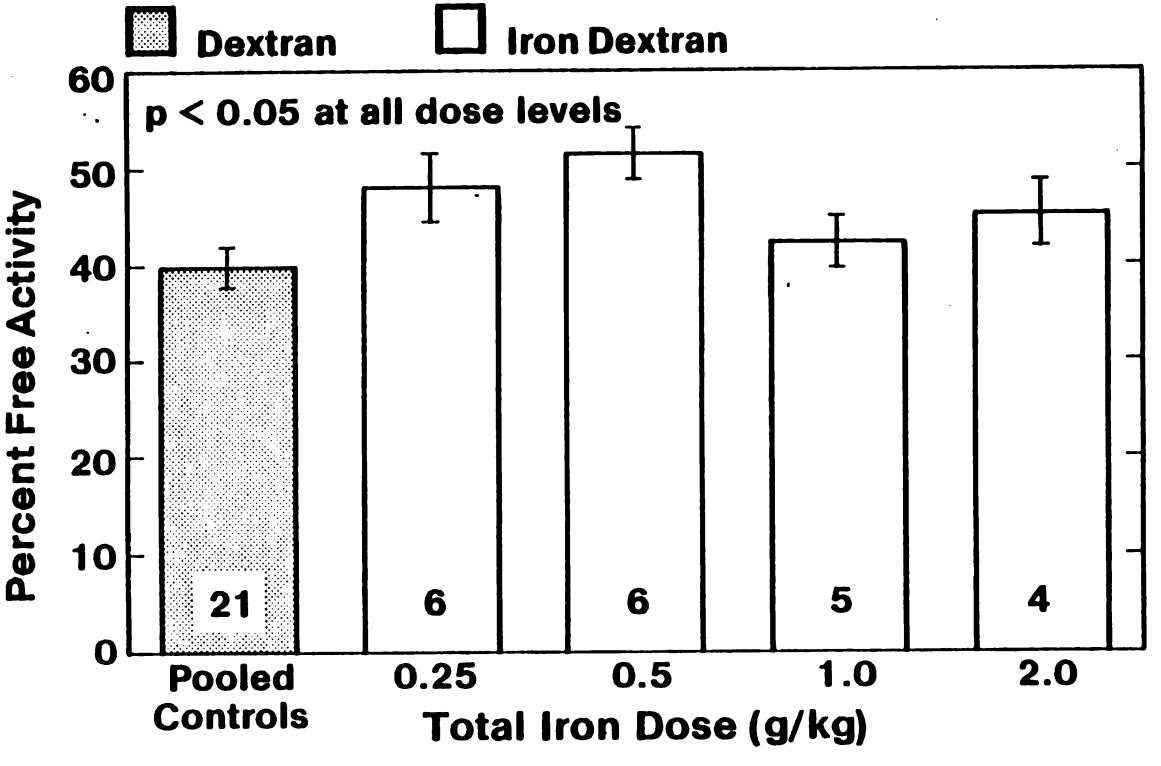

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Figure 8:

Comparison of Free Myocardial Acid Phosphatase in Pooled Dextran Controls vs. Iron Treated Guinea Pigs.

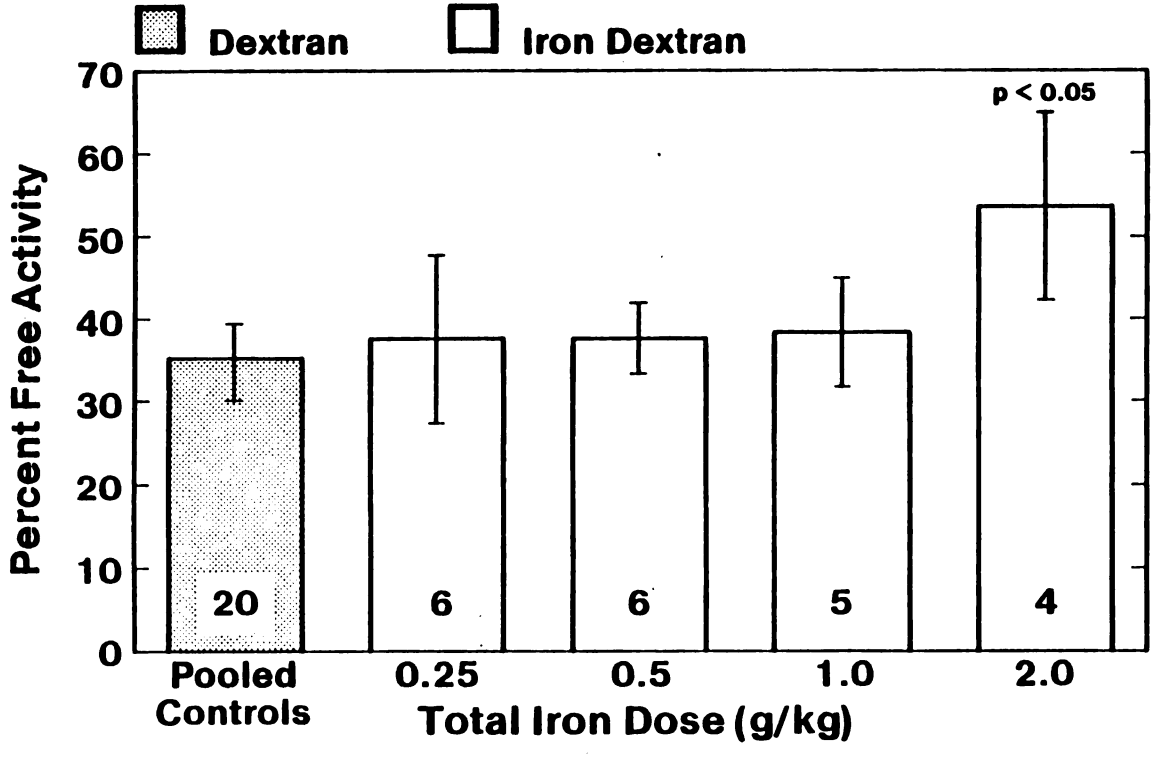

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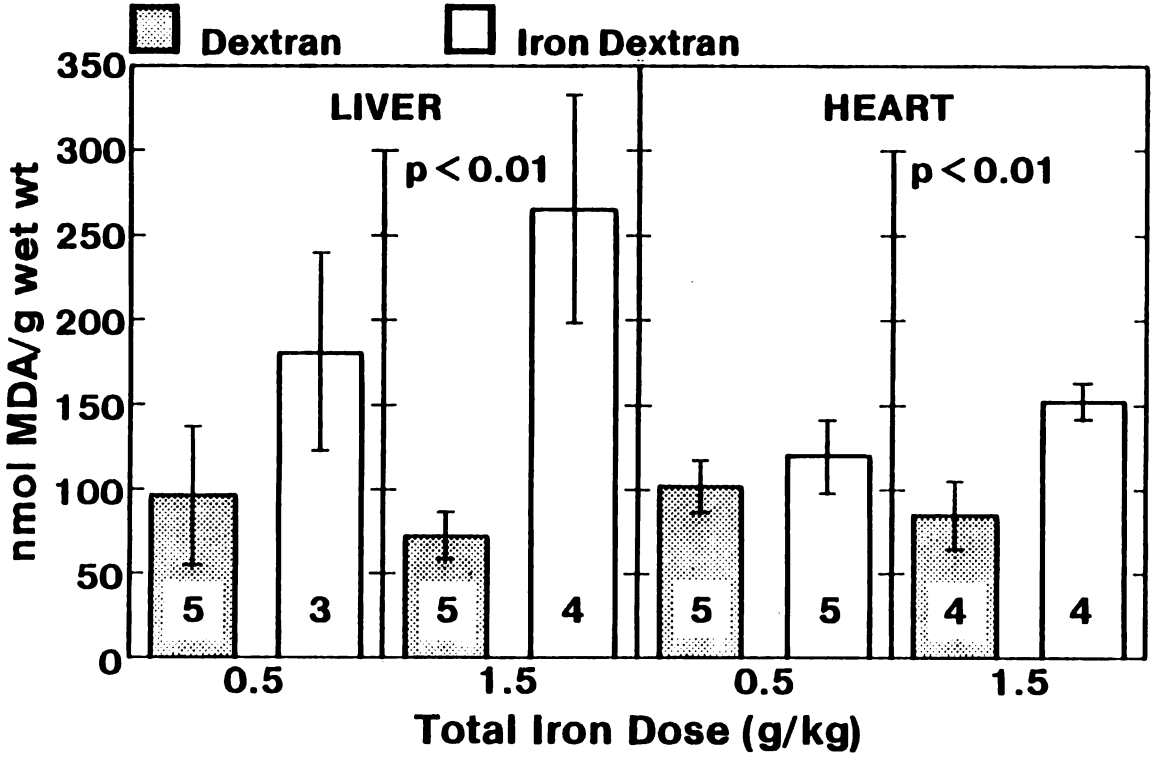
Figure 9

Comparison of Malondialdehyde (MDA) Levels in Whole Homogenates of Heart and Liver in Dextran Control and Iron Dextran Treated Guinea Pigs.



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lysosomes in human iron overload patients (1-4) and experimental animal models (5-8), myocardial lysosomes have largely been ignored. This study extends these previous reports by demonstrating similar lysosomal alterations in the myocardium.

In general, liver lysosomes demonstrated increased fragility at lower iron loads than myocardial lysosomes. Increases in free hepatic B-N-acetylglucosaminidase and B-glucuronidase followed a similar pattern and were suggestive of an initial maximal or near maximal release of enzyme at even the lowest iron dose level. A comparable response was shown with myocardial glucosaminidase, yet free myocardial acid phosphatase was demonstrated to be significantly elevated at only the highest level of iron loading, 2.0 g/kg. Variation in lysosomal enzyme analysis between the liver and heart may be partially explained by different cell types composing these tissues, and by the heterogeneity of lysosomal organelles and their response to different stimuli (20).

Demonstration of iron-enhanced lipid peroxidation in this report was consistent with numerous studies (7,21-24). Findings of a previous in vitro investigation indicated that incubation of isolated hepatic lysosomes in the presence of iron rapidly produced maximal levels of lysosomal lipid peroxidation with subsequent increase in lysosomal fragility (25,26). Similar results were demonstrated using mouse peritoneal macrophages (27). These studies established that lysosomal membranes were susceptible to peroxidation by both ferrous and ferric forms of iron, leading to the liberation of acid hydrolases. In addition, studies of cultured rat myocardial cells have demonstrated that iron loading results in (1) the rapid assimilation of large amounts of non-transferrin iron with an associated increase in lipid

peroxidation (28); (2) the accumulation of iron-filled ferritin molecules within endocytic vesicles and lysosomes (29); and (3) altered cellular contractions (30). The effects of iron loading were ameliorated by in vitro chelation with desferrioxamine; thus, directly implicating iron as the causative factor. These observations showed that cultured myocardial cells and most likely the lysosomes of these cells were susceptible to the toxic effects of iron. Our results in the iron loaded guinea pig model demonstrating increased membrane peroxidation and lysosomal enzyme release are comparable to these prior studies. The mechanism of iron-initiated peroxidation remain complex and largely unknown. However, it has been demonstrated that iron overload disorders result in "decompartmentalization" of stored iron leading to elevated levels of non-transferrin bound plasma iron (i.e., "free iron"); thus, potentially increasing the possibility of initiating iron-mediated membrane injury (31,32).

Determination of serum B-N-acetylglucosaminidase levels reflected tissue enzyme alterations. A previous study (33) demonstrated a linear correlation between serum NAG concentrations and iron stores (as determined by serum ferritin levels) in B-thalassemia intermedia patients. This correlation seemed to support the hypothesis that, in iron overloaded patients, lysosomal membranes are damaged resulting in increased permeability, release of lysosomal acid hydrolases and subsequent tissue injury. Interestingly, our results indicated that elevations in serum NAG appeared to follow increases in the free activity of hepatic NAG. Thus, this serologic assay may be useful as an indirect determinant of lysosomal fragility.

In summary, experiments with the iron loaded guinea pig model demonstrate: 1) serum iron and percent transferrin saturation are comparable with changes in humans with excess iron; 2) iron loading increases the fragility of both hepatic and myocardial lysosomes suggesting that lysosomes may perform an influential role in the pathogenesis of cardiomyopathy and hepatic disease commonly observed in iron overload disorders; 3) enhanced lysosomal fragility is likely the result of iron-catalyzed membrane peroxidation; 4) increases in serum glucosaminidase may be reflective of lysosomal damage.

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CHAPTER III

HISTOLOGIC AND ULTRASTRUCTURAL STUDIES IN THE IRON OVERLOAD GUINEA PIG MODEL

I. INTRODUCTION

Iron overload disorders, both acquired and inherited are associated with functional impairment of the liver, heart, and endocrine organs. Study of the pathophysiology of iron excess has been hampered by the lack of an easily reproducible animal model.

Previously developed animal models have been cumbersome, requiring up to 12 months of iron loading before experimentation can begin (1-4). Histological and ultrastructural descriptions of siderosis involving the liver of rats, as well as cultured neonatal rat myocardial cells have been published. However, morphological analysis of myocardial siderosis in an *in vivo* model has not been described.

Our prior studies using myocardial and hepatic tissue demonstrated abnormal lysosomal membrane stability and lipid peroxidation in guinea pigs receiving intraperitoneal injections of iron dextran. To correlate morphologic findings with these concurrent biochemical and functional studies we examined the histologic and ultrastructural features of hepatic and myocardial tissues in iron dextran treated guinea pigs.

II. MATERIALS AND METHODS

Animal Treatment

Female Hartley guinea pigs (Michigan Department of Public Health) weighing 200-250 grams were housed (3 per group) in stainless steel cages on wire grates and fed a commercial diet with vitamin C (Purina Guinea Pig Chow #5025, St. Louis, MO). Iron dextran (Sigma Co., St. Louis, MO) [83 mg Fe/kg/injection (pH 7.4)] was injected intraperitoneally on alternate days to achieve iron loading levels of 0.5, 1.0 and 1.5 g Fe/kg for histopathologic and ultrastructural evaluation. Controls received an equivalent quantity of dextran. All animals were sacrificed 48 hours following the final injection. Tissues (heart and liver) were perfused and fixed in 10% buffered formalin for histopathologic evaluation. Multiple sections of liver and heart were paraffin-embedded and stained with both hematoxylin/eosin and Prussian Blue. Portions of left ventricle were prepared for ultrastructural evaluation. Samples ($1mm^2$) were placed in an ice-cold fixative of 2% glutaraldehyde/2% paraformaldehyde in 0.1M $NaPO_4$ buffer (pH 7.4) for 3-4 hours. Tissues were washed several times in sodium phosphate buffer for 30 minutes to an hour and subsequently post-fixed with 2% OsO_4 in 0.1M $NaPO_4$, then dehydrated through a graded alcohol series. Following numerous washings, specimens were embedded in Spurr's low viscosity epoxy resin. Thin sections were cut on a LKB ultramicrotome-3 with glass knives, mounted on 300 mesh copper grids, left unstained or stained with saturated aqueous uranyl acetate and lead citrate, and viewed on a Philips 201 electron microscope operating at an acceleration of 60 kV.

X-Ray Microanalysis

For X-ray microanalysis, glutaraldehyde/paraformaldehyde fixed (postfixation with 2% OsO_4) left ventricular myocardial tissue was carbon-coated and left unstained. Analytical electron microscopy was performed using a JEOL 100 CX II with STEM attachment and a Link Systems AN 10000. The tissue was examined in the scanning transmission mode at 100 kV with a 30° tilt for 300 seconds.

III. RESULTS

Light Microscopy

No histologic changes except the presence of iron particles (This term is used throughout since the histologic and ultrastructural features of hemosiderin and ferritin are not well defined) were observed in sections of H & E stained myocardial and hepatic tissues. Sections of liver and heart from dextran treated controls were normal.

Liver

Prussian Blue staining demonstrated iron particles (IP) in sinusoidal lining cells (Kupffer cells, endothelial lining cells) in all areas of liver sections; neither preferential periportal nor centrilobular distribution of iron particles was observed. In guinea pigs administered 0.5 g Fe/kg, iron staining was observed exclusively within sinusoidal cells and not hepatocytes (Figure 10A). However, as iron loads increased IP within hepatocytes became clearly discernible, especially at the highest iron dose (Figure 10B).

Figure 10. Hepatic Siderosis

10A. Iron Dextran, liver, 0.5 g Fe/kg, guinea pig. Iron particles (arrows) confined to sinusoidal lining cells (mainly Kupffer cells) surrounding a central vein. Prussian Blue; Line = 100μm

10B. Iron Dextran; liver, 1.5 g Fe/kg, guinea pig. Positive staining iron particles are located within hepatocytes (arrows) and Kupffer cells (open arrows). Prussian Blue; Line = 50μm

Figures 10 A

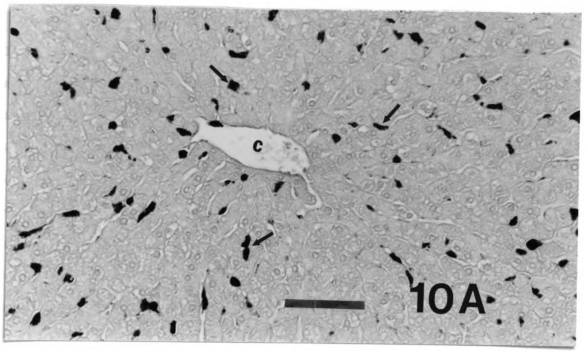
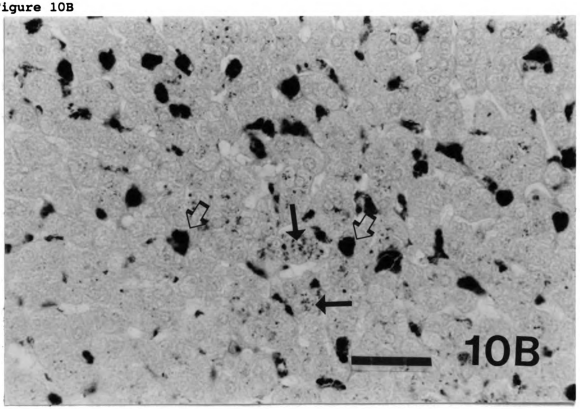


Figure 10B



Heart

Iron was demonstrable in all areas of the heart, particularly lining the epicardium (Figure 11A), endocardium (Figure 11B), and within macrophages of the subepicardial connective tissue (Figure 11C). Slightly lesser amounts of iron, located primarily in interstitial cells was observed in the myocardium (Figure 11D). Atrial myocardium also contained IP and the distribution was similar to that described for ventricular myocardium (Figure 11E). In addition, IP were found in sections of chordae tendineae and there was infrequent evidence of IP within cardiac myofibers (Figure 11F). Total quantity of iron increased moderately with increments of iron load.

Electron Microscopy

Ultrastructural evaluation was performed only on myocardial tissue from guinea pigs of the high dose group. Aggregates of electron dense, membrane-bound granules were occasionally observed within mesenchymal cells. These granules were primarily located perinuclearly (Figure 12) in lysosomal-like organelles (i.e., siderosomes) and were morphologically consistent with ferritin particles. In heavily loaded cells particles were abundantly free within the cytosol and not confined to membrane-delimited organelles. X-ray microanalysis of lysosomal structures within these cells confirmed that electron dense particles were iron (Figure 13A and 13B).

IV DISCUSSION

This study documents histologic and ultrastructural findings in hepatic and myocardial tissue of guinea pigs injected intraperitoneally

Figure 11A. Cardiac Siderosis

11A. Iron Dextran; atrium, 0.5 g Fe/kg, guinea pig. Iron particles lining epicardial surface (arrows). Prussian Blue, Line = 100μm

11B. Iron Dextran; Right Ventricle, 0.5 g Fe/kg, guinea pig. Iron particles lining the endocardium (arrows). Prussian Blue, Line = 50μm.

Figures 11 A

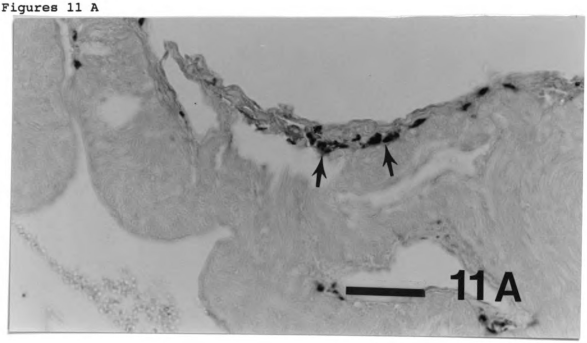
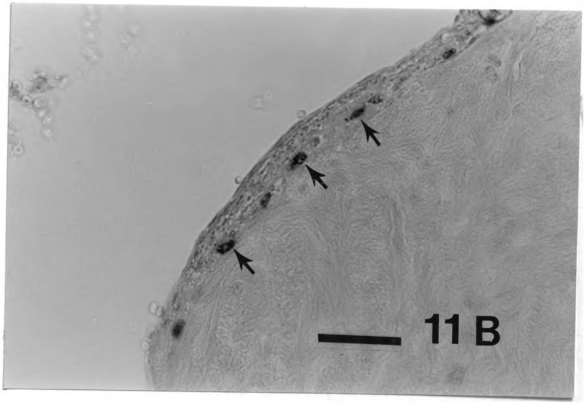


Figure 11 B



11C. Iron Dextran; subepicardial region, 1.5 g Fe/kg, guinea pig. Numerous intracellular iron aggregates within connective tissue macrophages (arrows) surrounding blood vessels (bv) and intermixed with collagen (open arrows). Prussian Blue, Line = 100μm.

11D. Iron Dextran; right ventricular myocardium, 1.5 g Fe/kg, guinea pig. Several aggregates of iron particles in interstitium of ventricular myocardium (arrows) between myocytes (*). Prussian Blue, Line = 100μm.

Figures 11 C

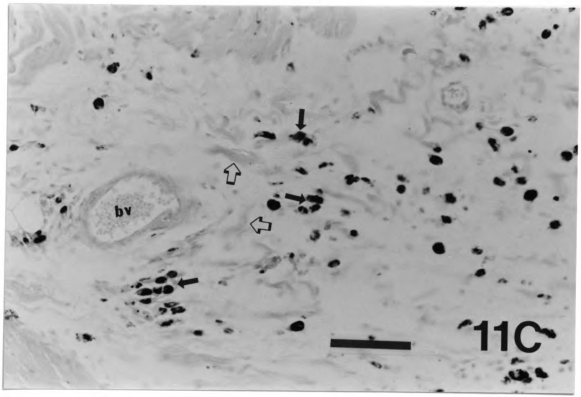
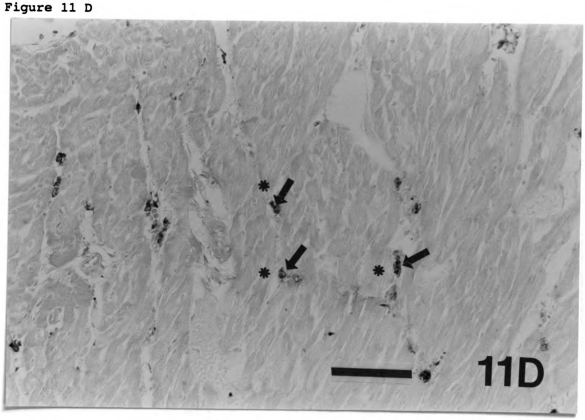


Figure 11 D



11E. Iron Dextran; atrial myocardium, 0.5 g Fe/kg, guinea pig. Iron aggregates in atrial interstitial tissue (arrows). Prussian Blue, Line = 50μm.

11F. Iron Dextran; right ventricular myocardium, 1.5 g Fe/kg, guinea pig. Iron particles in cardiac myofibers (arrows). Prussian Blue, Line = 100μm.

Figures 11 E

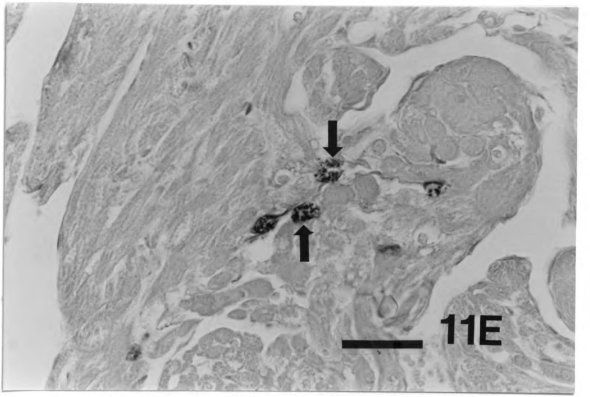
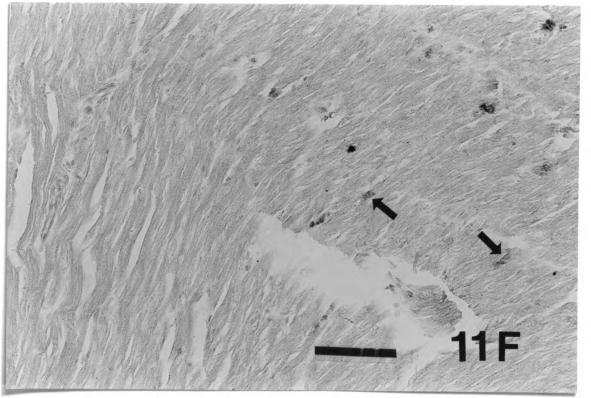


Figure 11 F



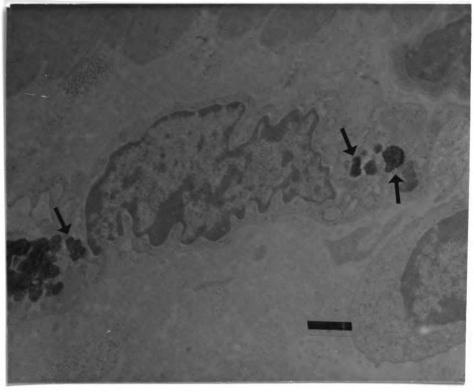
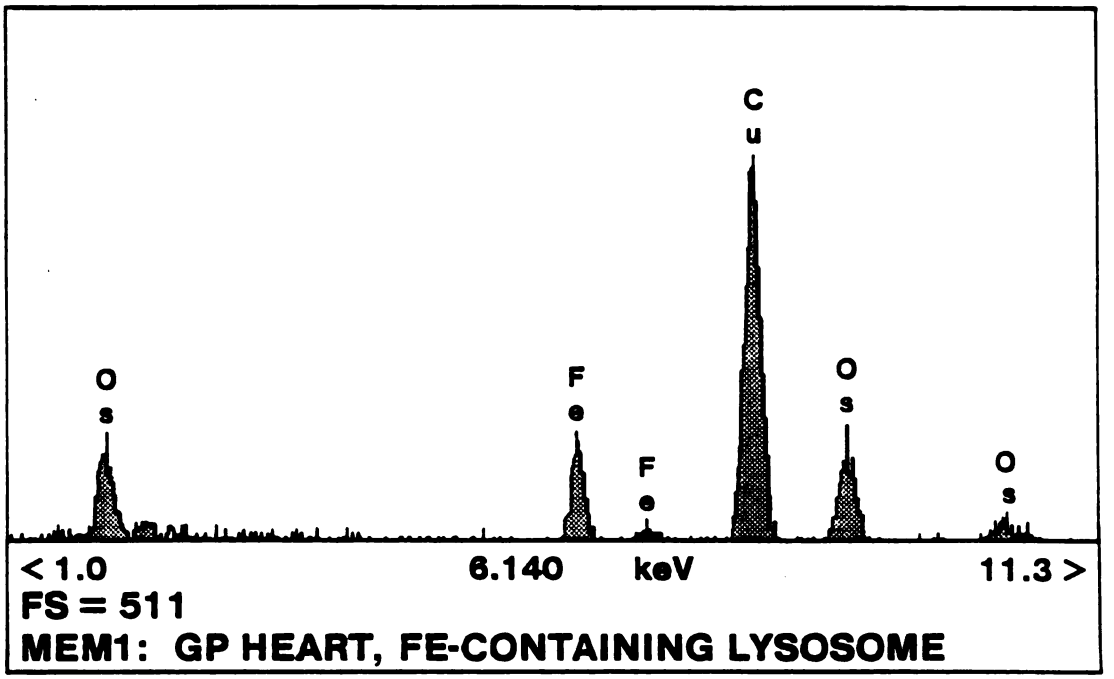


Figure 12. Interstitial cell with numerous perinuclear iron-filled lysosomes, i.e., siderosomes (arrows). Stained; Line = 500nm.

Figure 13A. Energy spectrum obtained from interstitial cell lysosomes (see electron micrograph Line = 1000nm; arrow) confirms the presence of iron (Fe). Copper (Cu) peaks are due to copper mesh grids; osmium (Os) peaks are the result of postfixation with OsO_4.

Figure 13A


Cu
Os
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< 1.0
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keV
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FS = 511
MEM1: GP HEART, FE-CONTAINING LYSOSOME

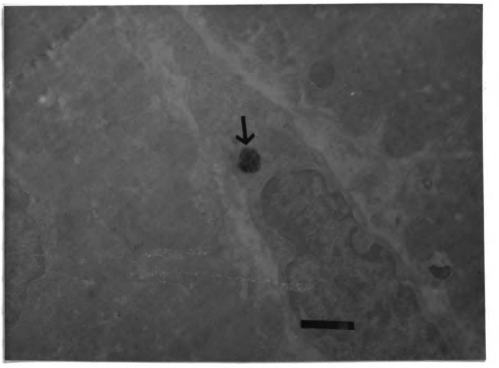

Figure 13B. Electron micrograph of cardiac interstitial cell with iron laden lysosome (arrow) - evaluated using STEM analysis (see energy spectrum in previous figure) Unstained; Line = 1000nm.

with iron dextran. The morphologic presence of iron complements the concurrent studies showing altered lysosomes and membrane peroxidation, thus helps to validate the guinea pig model for investigation of iron toxicity.

Previous attempts to create an animal model for iron overload have used various forms of iron, each differing in deposition sites. Injected iron-nitrilotriacetate (Fe-NTA) and dietary carbonyl iron preferentially accumulates in hepatocytes with minimal deposition within Kupffer cells, whereas iron dextran primarily deposits in Kupffer cells and to a lesser extent hepatocytes (3,5,6). In contrast, parenteral administration of Jectofer (iron-sorbitol-citric acid complex) leads to aggregation of iron particles in both parenchymal and Kupffer cells (1,7). Furthermore, these prior studies have demonstrated a periportal, as opposed to centrilobular distribution for carbonyl iron and Jectofer, similar to human hemochromatosis (1,5). Histologic findings in our study did not indicate a selective hepatic iron deposition pattern which is consistent with a previous report (6). Iron was initially confined to Kupffer cells and gradually extended to hepatocytes, a pattern morphologically more consistent with secondary, rather than primary forms of iron overload. These findings suggest that in this model, as in acquired forms of iron overload, parenchymal iron deposition does not occur until the iron retention capacity of the reticuloendothelial system is saturated.

Extensive studies on myocardial siderosis in an animal model have not been reported, however Iancu et al. (5) briefly described the presence of sparse ferritin particles within myocytes and endothelial cells in a carbonyl iron-fed rat model. Findings in this study show

that injection of iron dextran results in aggregation of iron particles in the heart of this model. The demonstration of interstitial cell siderosis (probably fibroblasts and connective tissue macrophages) suggests that in vivo there is an operative defense mechanism resulting in segregation of iron from myocytes. Nevertheless, results of parallel studies using this model show significant elevation in myocardial lysosomal membrane fragility with release of acid hydrolases, in addition to increased lipid peroxidation. It is important to note that iron-related release of lysosomal enzymes is thought to play a crucial role in the pathogenesis of tissue damage in hemochromatotic patients (8). Collectively, these related studies suggest that liberation of hydrolytic enzymes from the interstitial cells or possibly myocardial cells of the heart may initiate the cardiac abnormalities observed in this model. These findings are supported by prior reports demonstrating that in neonatal rat cardiac myocytes cultured in the presence of ferric ammonium citrate iron uptake is associated with sequestration of iron-filled ferritin into endocytic vesicles and lysosomes (9), increased intracellular concentrations of malondialdehyde, altered cellular contractility, and ultrastructural damage as evidenced by mitochondrial abnormalities and excessive autophagocytosis (5,10,11). Thus, in both studies iron loading is related to biochemical, morphologic and functional alterations.

Numerous clinical reports of myocardial hemosiderosis have described various histologic and ultrastructural changes in both primary, as well as acquired forms of iron overload. Consistent findings among these studies include (1) the presence of regional anatomic differences in ventricular iron deposition with the

subepicardial region containing the heaviest iron deposits; intermediate amounts subendocardially and lesser quantities in the midmyocardium (12-15); and (2) distinct patterns of intracytoplasmic iron distribution. Sanyal et al. (13) documented three patterns (1) paranuclear - a small collection of iron adjacent to the myofiber nucleus; (2) perinuclear - a more extensive deposition of iron around the nucleus; (3) diffuse - maximum quantities of iron present in a diffuse cytoplasmic pattern. These findings supported a previous study (12). Morphologic evaluation of hearts in this study reveal that iron particles are particularly prominent in epicardial and endocardial regions with slightly lesser amounts in the ventricular myocardium; similar findings are noted in atria. However, ultrastructural assessment clearly demonstrate that iron particles accumulate within membrane-bound structures around nuclei of mesenchymal cells.

In conclusion, this model mirrors many of the biochemical and morphologic findings documented in previous reports of experimental iron overload. Iron loading is consistently associated with the presence of iron particles in membrane-bound structures (siderosomes), enhanced lipid peroxidation, and fragile lysosomal membranes. The findings of this, as well as concurrent studies in our laboratory suggest the hypothesis that in the iron-loaded guinea pig, siderosis of myocardial interstitial cells may be the initial lesion leading to further biochemical and functional abnormalities.

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CHAPTER IV

BRIEF COMMUNICATION

Hypogonadism in Iron Loaded Male Guinea Pigs: A Preliminary Study

Hypogonadotropic hypogonadism, accompanied by loss of libido is a frequent endocrine dysfunction in men (1) with idiopathic hemochromatosis (IHC). The hypogonadism results from the selective deposition of iron in gonadotropic cells of the pituitary leading to decreased levels of plasma FSH/LH and testosterone (2). In a preliminary study, histologic evaluation of testicular tissue from iron dextran injected male guinea pigs (n=2) [83 mg Fe/kg/injection times 5 injection (pH 7.4) on alternate days from a total dose of 1.0 g Fe/kg] demonstrated moderate to severe atrophy of seminiferous tubules with decreased numbers of spermatogenic cells (Figure 14) when compared to dextran treated controls (Figure 15). Occasional intracellular aggregates of iron particles observed around interstitial (Leydig) cells of the testes, were absent in sections of pituitary gland. These results suggest that morphologic changes consistent with testicular atrophy observed in primary hemochromatosis may be reproduced with this model. Hypogonadism is particularly severe in young male patients affected with the juvenile form of IHC (3). Histologic studies from these patients indicate atrophic seminiferous tubules with scanty mitoses, absent spermatozoa and spermatids, thickening of the tubular walls and reduced numbers of Leydig cells (2). Similar findings were observed in this brief investigation. Future studies with this model

would be enhanced by using morphometric techniques and assessing endocrine function, i.e., determining plasma levels of FSH, LH and testosterone. In addition, analysis of response to hypothalamic stimulation would be of value.

Figure 14

Photomicrograph of testicle from iron dextran treated male guinea pig with atrophy of seminiferous tubules. Note lack of normal maturation and mitotic activity of spermatogenic epithelium; marked vacuolation is notable. Hematoxylin & eosin; Line = 100μm.

Figure 15

Photomicrograph of testicle from dextran treated control male guinea pig. Maturation of spermatogenic cells from spermatogonia (arrows) to spermatids (open arrows) is normal. Hematoxylin and eosin; Line = 100μm.

Figure 14

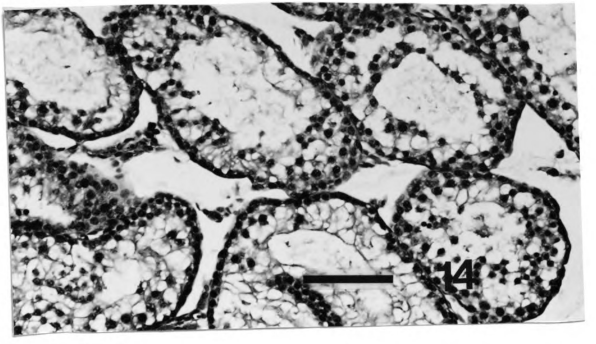
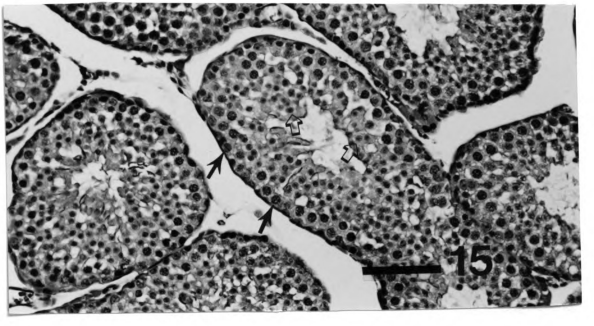


Figure 15



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APPENDICES

APPENDIX A: Specific Activity of Hepatic and Myocardial B-N-acetylglucosaminidase (NAG) in Dextran Control and Iron Treated Guinea Pigs

IRON DOSAGE		HEPATIC NAG	MYOCARDIAL NAG
0.25	Con	11.48 ± 1.859	7.0195 ± 0.2782
	Fe	12.41 ± 1.91	7.929 ± 1.094
0.5	Con	14.37 ± 1.088	8.525 ± 0.7998
	Fe	12.62 ± 1.575	* 9.669 ± 0.7126
1.0	Con	12.5 ± 2.685	8.502 ± 1.117
	Fe	** 19.84 ± 2.38	* 11.42 ± 0.2256
2.0	Con	15.48 ± 3.67	7.42 ± 0.8871
	Fe	13.95 ± 2.082	7.275 ± 0.6396

NAG units = nmol p-nitrophenol/ug protein/min ($\times 10^{-3}$)

Dextran Control = Con

Iron Treated = Fe

* $p < 0.05$

** $p < 0.01$

APPENDIX B: Specific Activity of Hepatic and Myocardial Acid Phosphatase in Dextran Control and Iron Treated Guinea Pigs

IRON DOSAGE		HEPATIC ACID PHOSPHATASE	MYOCARDIAL ACID PHOSPHATASE
0.25	Con	3.024 ± 0.7271	1.301 ± 0.0697
	Fe	4.808 ± 1.462	1.156 ± 0.1052
0.5	Con	4.527 ± 0.4451	1.872 ± 0.3196
	Fe	** 2.874 ± 0.5877	1.773 ± 0.296
1.0	Con	4.27 ± 1.168	1.542 ± 0.266
	Fe	5.09 ± 0.9822	1.8282 ± 0.33
2.0	Con	8.429 ± 1.098	1.623 ± 0.2084
	Fe	* 12.572 ± 1.67	** 3.246 ± 0.1241

Dextran Control = Con

Iron Treated = Fe

Acid Phosphatase expressed as

(nmol PO_4 /ug protein/min (x 10^{-3})

* $p < 0.01$

** $p < 0.001$

APPENDIX C: Specific Activity of B-glucuronidase in Hepatic Tissue of Dextran Control (Con) and Iron Treated (Fe) Guinea Pigs.

Iron Dose		Hepatic B-glucuronidase
0.25	Con	2.49 ± 0.5748
	Fe	2.818 ± 0.5781
0.5	Con	2.517 ± 0.5311
	Fe	2.307 ± 0.4662
1.0	Con	2.88 ± 0.4605
	Fe	3.553 ± 0.6292
2.0	Con	2.865 ± 0.7351
	Fe	2.489 ± 0.3484

B-glucuronidase expressed as
(nmol p-nitrophenol/ug protein/min ($\times 10^{-3}$)
No significant differences noted.

Appendix D

Serum Iron (SI), Total Iron Binding Capacity (TIBC) and Transferrin Saturation (Tf Sat) Values in Control Guinea Pigs

Iron Dose (g/kg)	Animal No.	SI (ug/dl)	TIBC (ug/dl)	Tf Sat (%)
	8-1	304	298	102.01
	8-2	228	291	78.35
0.25	8-3	236	236	100.00
(n=4)	8-4	215	274	78.46
		245.8±34.5	274.75±27.72	87.70±13.07
	4-1	265	267	99.25
	4-2	281	290	96.89
0.5	4-3	240	233	103.00
(n=6)	4-4	296	310	95.48
	4-5	288	276	104.34
	4-6	233	265	87.92
		267.16±25.94	273.5±25.94	97.81±5.92
	0-1	259	–	–
	0-2	291	303	96.03
1.0	0-3	333	322	103.41
(n=6)	0-4	273	278	98.2
	0-5	295	295	100.00
	0-6	319	354	90.11
		295.0±27.62	310.4±29.05 (n=5)	97.55±4.96 (n=5)

Appendix E

Serum Iron (SI), Total Iron Binding Capacity (TIBC) and Transferrin Saturation (Tf Sat) Values in Iron Dextran Treated Guinea Pigs

Iron Dose (g/kg)	Animal No.	SI (ug/dl)	TIBC (ug/dl)	Tf Sat (%)
	7-1	315	270	116.66
	7-2	285	251	112.35
0.25	7-3	252	232	108.62
(n=6)	7-4	269	251	107.17
	7-5	234	218	107.33
	7-6	218	231	106.92
		266.5±26.6	242.16±18.66	109.84±3.9
	3-1	689	745	92.48
	3-2	1074	1194	89.94
0.5	3-3	950	1066	89.11
(n=6)	3.4	1141	1200	95.08
	3-5	511	588	86.9
	3-6	660	930	70.96
		837.5±253.32	953.83±248.54	87.41+8.54
	1-1	585	498	117.46
	1-3	1313**	–	–
1.0	1-4	591	492	120.12
	1-5	321	–	–
	1-6	398	362	109.94
		473.75±135.64 (n=4)	450.66±76.84 (n=3)	115.84±5.27 (n=3)

** = outlier

Appendix F

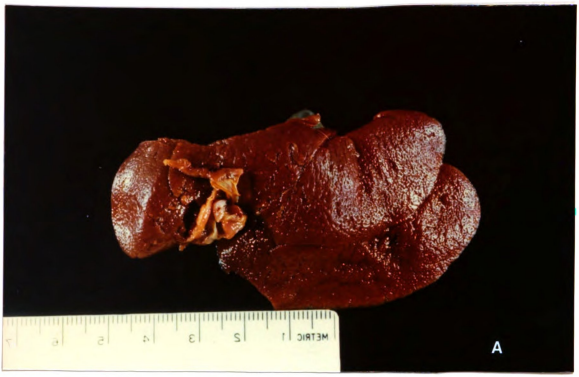
Serum B-N-acetylglucosaminidase (NAG) activity (expressed as nmoles p-nitrophenol/min/ug protein {$x10^{-3}$}) in Dextran Control and Iron Dextran Treated Guinea Pigs

	Control		Fe Treated	
Iron Dose (g/kg)	Animal No.	Activity	Animal No.	Activity
0.25	8-1	2.584	7-1	4.383
	8-2	3.802	7-2	6.96
	8-3	3.277	7-3	8.195
	8-4	3.015	7-4	6.644
			7-5	8.491
			7-6	10.9
		3.1695±.5093		7.593±2.177
0.5	4-1	4.059	3-1	9.802
	4-2	3.561	3-2	9.085
	4-3	3.584	3-3	7.035
	4-4	3.583	3-4	7.655
	4-5	4.225	3-5	6.239
	4-6	3.709	3-6	8.344
		3.786±.2848		8.026±1.318
1.0	0-1	3.073	1-1	14.33
	0-2	3.011	1-2	17.42
	0-3	3.09	1-3	17.36
	0-4	3.094	1-4	16.16
	0-5	3.155	1-6	20.14
	0-6	2.97		
		3.065±.0656		17.08±2.119
2.0	6-1	2.717	5-1	11.7
	6-2	2.664	5-2	7.285
	6-3	2.624	5-4	8.754
	6-4	2.786	5-5	7.06
	6-5	3.365		
		2.831±.3045		8.699±2.136

Appendix G

A. Gross photograph of liver from septic guinea pig. Note granular, jaundiced appearance of hepatic surface with numerous, multifocal regions of necrosis.

B. Gross photograph; cross section of liver from septic guinea pig demonstrates multiple regions of necrosis.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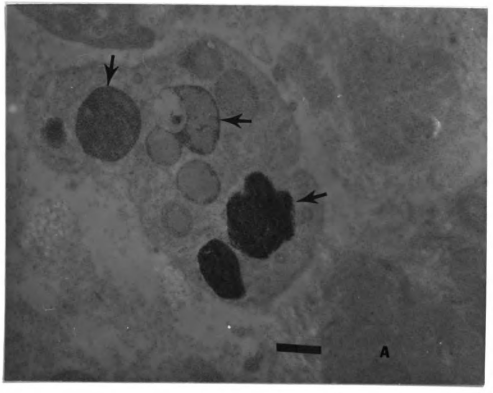
Appendix H

Electron micrographs of cardiac interstitial cells from iron dextran treated guinea pigs (1.5 g/kg). Tissues were fixed with 2% glutaraldehyde/2% paraformaldehyde and postfixed with 2% OsO_4; thin sections were stained with saturated aqueous uranyl acetate and lead citrate or left unstained.

A. **Siderosomes containing variable amounts of iron (arrows). Unstained; Magnification x 23,000**

B. **Interstitial cell containing abundant iron particles within lysosomes (arrows) and extending beyond lysosomal membrane boundaries (arrowheads). Unstained; X 20,000.**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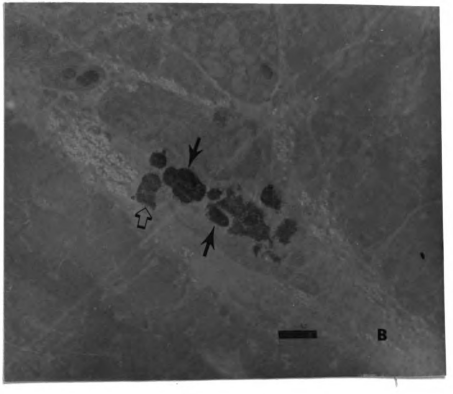
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