CELLULAR IRON METABOLISM
IN HAEMOCHROMATOTIC MACROPHAGES

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DECLARATION

This dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. No part of it has been presented for any degree or examination at any other University.

C. ICKINGER

ABSTRACT

HLA-linked haemochromatosis is the result of an inborn error of metabolism inherited as an autosomal recessive gene, closely linked to the HLA locus on chromosome six. In this condition iron absorption is inappropriately high leading to iron overload. Integral to the pathogenesis of this disorder and in contrast to other causes of iron overload, is the relatively modest accumulation of iron within cells of both the small intestine and the reticuloendothelial system and the excessive deposition of iron in parenchymal cells of the liver and other organs. This observation has led to the suggestion that the primary defect(s) could be present in either the gut, the liver, the reticuloendothelial system or all three. Abnormalities in iron uptake by cells, iron transport through and between cells and iron storage in cells have all been suggested as possible mechanisms responsible for the abnormal absorption and distribution of iron in haemochromatosis. Malfunction of the iron transport protein transferrin or its receptor could be responsible for abnormal distribution and iron loading while an abnormality of ferritin iron storage could explain why some cells appear to be unable to store iron and others are iron overloaded. Major advances have recently been made in our understanding of both ferritin synthesis and transferrin receptor expression. It is now known that when cellular iron levels are low, ferritin

synthesis and iron storage decrease, while transferrin receptor synthesis and iron uptake increase. Conversely, iron in excess of cellular requirements results in an increase in ferritin synthesis and a decrease in transferrin receptors. This regulation occurs at the level of mRNA translation, mediated by the interaction of iron regulatory proteins and iron responsive elements on the respective mRNAs. Despite considerable study of the molecular basis underlying the reciprocal and coordinated regulation of iron transport into cells and iron storage, little is known about the mechanisms by which cells release iron.

This study evaluates certain aspects of iron metabolism in relation to the macrophage in hereditary haemochromatosis. Macrophages cultured for 8 days *in vitro* were first assessed for viability by trypan blue dye exclusion, tritiated uridine and tritiated leucine incorporation into RNA and protein, respectively. These experiments revealed that the cells from both normal subjects and patients with hereditary haemochromatosis remained metabolically viable after 8 days in culture.

A study was done to evaluate the uptake of transferrin and transferrin iron by 8 day cultured macrophages obtained from normal subjects and treated haemochromatotic patients. A 1.9 fold and 1.4 fold increase in transferrin uptake by haemochromatotic cells over normal cells was noted at 1 hour ($p = 0.003$) and at 1.5 hours ($p = 0.009$), respectively. However, iron uptake by haemochromatotic

macrophages was 2.7 times lower at two hours ($p = 0.01$) and 2.1 times lower at five hours ($p = 0.006$) than normal macrophages. The results suggest an increased transferrin receptor expression in macrophages from patients with haemochromatosis. The failure of iron uptake to parallel transferrin uptake in haemochromatotic cells could represent an inability to sequester iron in the storage protein ferritin or an increased cellular iron release by some other unknown mechanism.

In the next study, RNA was extracted at various time intervals from normal and haemochromatotic macrophages cultured in the presence of ferric iron. The RNA was probed for H- and L-ferritin subunit mRNA with ß-actin acting as a constitutively expressed control. Although the levels of H-and L-ferritin mRNA relative to ß-actin increased in both study groups, the increase in ferritin subunit mRNAs was about 4 to 5 fold in normal macrophages while in haemochromatotic macrophages a 1.5 to 2 fold increase was observed after six hours. The regulation of ferritin synthesis by iron is known to occur at both transcriptional and translational levels. The greater increase in ferritin subunit mRNAs observed in normal macrophages suggests that, in haemochromatotic macrophages, the mechanism of iron-induced mRNA expression may be impaired by changes in either transcription or translation. Alternatively, the decreased ferritin expression may occur as a secondary event in response to lower intracellular iron, possibly caused by accelerated

release of iron from haemochromatotic cells.

In summary, the findings of these investigations suggest that the paucity of iron within reticulo-endothelial cells in haemochromatosis may result from a primary defect in ferritin mRNA expression or alternatively from a secondary decrease in cellular iron storage because of enhanced iron release. These experiments were conducted on a small number of patients and were confined to specific steps in cellular iron metabolism. Further work, using more patients and studying the distribution of free to polysome-bound mRNA, the rate of ferritin mRNA turnover and the amount of the final protein product, ferritin, would be required in order to fully address ferritin synthesis in haemochromatotic macrophages.

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SECTION 1

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

HLA-linked (idiopathic, genetic, hereditary, primary) haemochromatosis is an iron storage disorder in which there is an increase in total body iron. The increased deposition of the metal in parenchymal cells of the liver, heart, pancreas and other organs occurs as a result of unexplained excessive mucosal absorption of dietary iron and increased release of iron from the reticuloendothelial (RE) system. The prolonged presence of large amounts of iron within the parenchymal cells eventually results in organ dysfunction (Bothwell et al, 1995). A brief description of some basic concepts of normal iron metabolism is required as a background to understanding this disease.

Iron is an essential element required for metabolic processes in cells and microorganisms (Crichton and Charloteaux-Wauters, 1987). A healthy adult male has approximately 3 to 4 grams of body iron (Bothwell et al, 1979). The largest portion of body iron is found in the functional iron-containing proteins haemoglobin, myoglobin, haem and non-haem enzymes (Smith, 1990). The iron porphyrin complex of haemoglobin and myoglobin serves to transport oxygen to cells and to store oxygen within tissues, respectively. The various haem enzymes (such as cytochromes, catalases, peroxidases) and non-haem iron proteins are involved in the electron transport pathway and other oxidation and reduction pathways (Pippard and Hoffbrand, 1989). Most of the remaining iron (about 0 to 1 gram depending on the individual's iron status) is found

in the iron storage protein ferritin and in haemosiderin. Ferritin is the main iron store, but in iron overload an increasing proportion is found in haemosiderin. Storage iron is distributed in the RE cells of the bone marrow, liver, spleen and skeletal muscle, mainly as haemosiderin and in hepatic parenchymal cells mostly in the form of ferritin (Bothwell et al, 1995). The carrier plasma protein transferrin is responsible for ferrying iron between these tissues.

Iron exists predominantly in the oxidized state (ferric form) which is insoluble and therefore largely inaccessible (Bothwell et al, 1979). Much of the dietary iron, although plentiful, is unavailable and iron metabolism, in humans, is characterized by conservation of body iron achieved by a continuous recycling process. The normal plasma iron level is about 3 mg. All of this iron is bound to transferrin. About 30 to 40 mg of iron enters and leaves the plasma pool per day (Cook et al, 1970; Ricketts et al, 1977; Cazzola et al, 1985). The maintenance of this iron pool is effected by internal and external iron exchange pathways.

Internal Iron Exchange. Most of the iron entering the plasma iron pool is derived from catabolism of erythrocyte haemoglobin by RE cells (Morgan, 1981). Other sources of plasma iron are derived from storage sites such as the liver, muscle and skin and from dietary iron absorbed from intestinal mucosal cells and a small amount from the extravascular pool. The major flow of iron from plasma is

delivered to erythropoietic tissue via the transport protein, transferrin, for the formation of haemoglobin (Morgan, 1981; Huebers and Finch, 1987). Some of this iron entering the erythron is not used for erythropoiesis, but is intercepted by the RE cells. The process is termed "ineffective" erythropoiesis (Bezkorovainy, 1989). Plasma iron is also exchanged with parenchymal cells of the liver and with the extravascular pool (Bothwell et al, 1979). The liver hepatocyte plays a pivotal role in iron metabolism in that it is capable both of releasing iron when there is an increased need in other tissues and of taking up excess iron when the transferrin saturation (iron-binding capacity) is increased.

External Iron Exchange. This involves iron gained from and lost to the environment. The obligatory daily loss of iron in adult males amounts to about 1 mg (Green et al, 1968). This is derived from the gastrointestinal and urinary tracts and skin. Iron losses are increased through menstruation and pregnancy in females. Requirements are also higher during periods of rapid growth in infancy and adolescence (Bothwell et al, 1979). To maintain the equilibrium, an equal amount to that lost to the exterior has to be absorbed from the daily diet. Mucosal iron absorption depends largely on the state of body iron stores. Iron absorption is decreased when iron stores rise and is increased when stores are depleted (Cook et al, 1974; Walters et al, 1975b; Charlton et al, 1977; Bezwoda et al, 1979). The mechanisms involved in the regulation of iron absorption have not yet been clarified.

1.2 IRON TRANSPORT - TRANSFERRIN

1.2.1 Introduction

The low solubility of ferric ions in physiological fluids and the potential toxicity of free iron necessitates the presence of specific iron complexing agents. The transferrins are glycoproteins capable of reversibly binding iron. These proteins are widely distributed in biological fluids ; the three major types comprise the serotransferrins (in plasma and extracellular fluid), the ovotransferrins (in egg whites) and the lactotransferrins (in extracellular secretions and leucocytes) (de Jong et al, 1990). The main function of serum transferrin is the transport of iron from cells involved in absorption (intestinal mucosal cells), storage (hepatocytes and RE cells) and erythrocyte catabolism to cells requiring iron for their metabolism. Important sites to which transferrin delivers iron are the developing erythroid cells of the bone marrow and the placenta (Aisen and Brown, 1977). The cellular uptake of transferrin-bound iron is brought about by specific membrane receptors. The mechanism by which cells deliver their iron to transferrin is less clearly understood. In the sections that follow the structural characteristics of transferrin and the transferrin receptor and the regulation of their synthesis in relation to iron will be discussed. The uptake of iron from and the release of iron to transferrin by cells will also be dealt with here.

1.2.2 Structure

Human transferrin is a beta-1 globulin glycoprotein of molecular weight 80 kDa. It consists of a single polypeptide chain composed of 679 amino acid residues and two N-linked complex glycan chains (Karlsson et al, 1978; MacGillivray et al, 1983). The molecule consists of two homologous domains, an N-terminal domain and a C-terminal domain. Each domain is capable of binding one atom of Fe(III) and requires the presence of an anion (bicarbonate or carbonate) (Bezkorovainy, 1989). Plasma transferrin is normally about one third saturated with iron. Four different forms of transferrin, depending on its degree of iron saturation, can be distinguished. They are diferric- (2 iron atoms bound), monoferric N- (iron in the N-domain), monoferric C- (iron in the C-domain) and apotransferrin (no iron bound) (Makey and Seal, 1976). It has been shown that diferric transferrin donates iron more efficiently to growing and dividing cells than monoferric transferrin (Morgan, 1981; Huebers et al, 1983).

1.2.3 Synthesis

The transferrin gene is located on chromosome 3 at q21-25 (Yang et al, 1984). The major source of plasma transferrin is the liver, but other tissues involved in the synthesis of this protein are the brain, heart, spleen, kidney, muscle and mammary gland (Aldred et al, 1987; Lee et al, 1987; de Jong et al, 1990). The synthesis of transferrin is inversely related to iron stores. The

concentration of transferrin is increased in iron deficiency and is decreased in iron overload (Lipschitz et al, 1974). The mechanisms of transcriptional and translational regulation of transferrin synthesis by iron have not been elucidated. However, a number of metal regulatory elements have been identified in the promoter region of the gene (Adrian et al, 1986; Hamer, 1986; Schaeffer et al, 1989). Plasma transferrin concentrations are also increased in pregnancy and during oestrogen administration (Bothwell et al, 1979). Furthermore, plasma transferrin levels may fall during the acute phase response in various inflammatory states, in myocardial infarction and malignant neoplasms (Bothwell et al, 1979).

1.2.4 Use as diagnostic index

Serum transferrin is capable of binding approximately 60 μmol Fe/l. However, under physiological conditions transferrin is only about 30% saturated (Bothwell et al, 1995). The serum iron and, more importantly, the saturation of the iron binding capacity gives an indication of the iron supply to tissues. The total iron binding capacity (TIBC) depends on the concentration of transferrin and is inversely related to the plasma iron levels. In pregnancy and iron deficiency anaemia, the transferrin levels increase (and therefore the TIBC) with a concomitant drop in the transferrin saturation (Morgan, 1981), favouring iron transfer from stores to plasma. In conditions such as hereditary haemochromatosis, high

plasma iron levels are associated with a high percentage transferrin saturation even before body iron is increased (Bassett et al, 1984). It follows that the estimation of saturation of the serum TIBC is important in assessing iron overload, to be discussed in chapter 1.5.4.

1.2.5 Transferrin receptors

1.2.5.1 Introduction

The transfer of iron from transferrin to cells involves the binding to specific receptor sites located on the cell membrane. Transferrin receptors are present on virtually all cell types particularly on dividing, differentiating and haemoglobin synthesizing cells (Huebers and Finch, 1987; Bothwell et al, 1995). The expression of transferrin receptors appears to depend on the iron status of cells. A process of receptor mediated endocytosis facilitates the cellular uptake of iron.

1.2.5.2 Structure

The transferrin receptor is a transmembrane glycoprotein consisting of two monomers of molecular weight 90 kDa, linked as a dimer by a disulphide bridge (Omary and Trowbridge, 1981; Schneider et al, 1982). Each subunit is able to bind one transferrin molecule (Enns and Sussman, 1981).

1.2.5.3 Synthesis

The gene for the human transferrin receptor is found on chromosome 3q21-26 (Miller et al, 1983; Rabin et al, 1985). The primary structure has been determined by

cloning and sequencing (Kuhn et al. 1984; McClelland et al. 1984; Schneider et al. 1984). The regulation of transferrin receptor expression is complex. The number of receptors on the cell surface appears to be influenced by many factors. There are clear indications that there is a strong correlation between cellular proliferation and the levels of transferrin receptor expression (Trowbidge and Shackelford, 1986). It is generally high in rapidly proliferating cells. Barnes and Sato (1980) provided further evidence that deprivation of tissue culture cells for transferrin, or blocking of the transferrin receptor with specific monoclonal antibodies (Trowbidge and Lopez, 1982), leads to inhibition of cellular proliferation. It therefore appears that the transferrin receptor is involved not only in delivering iron to cells but also plays a direct role in the regulation of cell growth, an effect that has been difficult to substantiate.

The main factor though controlling the expression of transferrin receptors is the intracellular chelatable iron pool. Evidence to support this finding was initially provided by studies in which cells treated with permeant iron chelators (Bridges and Cudkowicz, 1984; Testa et al. 1985) or grown in iron deficient media (Rudolph et al. 1985), resulted in an up-regulation of transferrin receptors. In contrast, exposure of a variety of cells to agents that supply iron (such as haemin or iron salts) resulted in decreased receptor numbers (Testa et al. 1982; Ward et al. 1982a & b; Ward et al. 1984b). The effects can be accoun-

ted for by altering the rate of synthesis of the receptors (Bomford and Munro, 1985). A negative feedback is in operation : when the available intracellular iron levels are low, the number of transferrin receptors increase to acquire iron more effectively and when the cells accumulate iron, the transferrin receptors decrease to limit further iron uptake. This modulation of transferrin receptor expression by iron has been confirmed by numerous other studies (Louache et al, 1984; Mattia et al, 1984; Rao et al, 1985; Muller-Eberhard et al, 1988). It has further been shown that transferrin receptor mRNA levels are increased when iron availability is low and decreased when iron is plentiful (Mattia et al, 1984; Mullner and Kuhn, 1988). It was demonstrated by Mullner and Kuhn (1988) that this effect was due to changes in the stability of the transferrin receptor mRNA, effected by a post-transcriptional mechanism.

The regulation of transferrin receptor expression by iron is closely coordinated with the expression of ferritin (Harford and Klausner, 1990). In contrast to the transferrin receptors, ferritin synthesis is increased without a corresponding change in the level of ferritin mRNA in the presence of elevated intracellular iron concentrations (Zahringer et al, 1976a; Aziz and Munro, 1986; Rogers and Munro, 1987). The increased synthesis is mainly due to an increase in translation of the ferritin mRNA, as opposed to a decrease in stability of transferrin receptor mRNA observed during iron loading. The molecular

mechanisms for this coordinate but opposite regulation will be fully discussed in chapter 1.3.6 under 'ferritin gene regulation and synthesis.'

1.2.6 Transferrin-iron-cell interactions

1.2.6.1 Iron uptake from transferrin

There is now good evidence showing that cells acquire iron from transferrin by a process of receptor mediated endocytosis. The model has been extensively discussed in several reviews (Crichton and Charlotaeux-Wauters, 1987; Thorstensen and Romslo, 1990; Pollack, 1992; Baker and Morgan, 1994). The mechanism has been demonstrated in reticulocytes (Harding et al, 1983), hepatoma cells (Dautry-Varsat et al, 1983) and erythroleukaemic cells (Klausner et al, 1983). The process is initiated by binding of diferric transferrin to the transferrin receptor. The interaction between transferrin and its receptor is calcium dependent (Hemmaplardh and Morgan, 1977) and maximal at a pH of 7,8 (van Bockxmeer et al, 1978) on reticulocytes. The receptor is concentrated in clathrin coated pits. Clathrin is a membrane protein that supports receptors for a variety of molecules. The whole receptor ligand is internalized by invagination of these coated pits to form endocytic vesicles termed endosomes. These vesicles are transported along microtubule and microfilament tracts by saltatory motion in the cell cytoplasm (May and Cuatrecasas, 1985). The vesicle's lumen is rapidly acidified to pH 5,5 by an energy-dependent

proton pump (van Renswoude et al, 1982). The acidified vesicle is known as the compartment of uncoupling of receptor and ligand (CURL) (Goldstein et al, 1979). The low pH facilitates the dissociation of iron from transferrin. The mechanism by which iron is transferred across the membrane of the endosome to the cytoplasm is still unclear. Several studies have shown that iron, probably as Fe(II), is actively transported across the membrane by a membrane-bound protein (Egyed, 1988; Fuchs et al, 1988; Morgan, 1988). It was suggested by Richardson and Baker (1992), that a temperature-dependent membrane protein may play a role in the transport of iron across the membrane of the endocytic vesicle. The apotransferrin-receptor complex, unlike other ligand-receptor complexes that are completely degraded within lysosomes, remains stable within the acidic microenvironment of CURL (Klausner et al, 1983). The iron-free apotransferrin bound to its receptor is then recycled back to the cell surface. At the extracellular pH of 7,0-7,4 the apotransferrin dissociates from the receptor and both molecules can be reutilized again.

The process of receptor mediated endocytosis is not the only mechanism whereby cells take up iron from transferrin. Alternative pathways have been demonstrated in hepatocytes. These include a receptor mediated endocytic pathway effective at low transferrin concentrations in combination with non-saturable transferrin binding at high transferrin concentrations (Page et al, 1984), fluid phase

endocytosis (Sibille et al. 1982; Trinder et al. 1986) and a non-specific asialoglycoprotein receptor pathway (Regoeczi and Koj, 1985).

Finally, although virtually all plasma iron is bound to transferrin, a number of compounds including haptoglobin, haemopexin, ferritin and low molecular weight plasma iron complexes constitute the non-transferrin-bound iron pool. In patients with marked iron overload, saturation of the binding capacity of transferrin results in the presence of higher concentrations of low molecular weight iron complexes (Batey et al. 1980; Hershko and Peto, 1987; Grootveld et al. 1989) and ferritin (Pootrakul et al. 1988) in the plasma. Non-transferrin-bound iron is potentially toxic and this may play an important role in causing the organ damage associated with iron overload.

1.2.6.2 Iron release from cells

To maintain the plasma iron pool the amount of iron taken up by cells has to be balanced by an equal amount returning to the plasma (Morgan, 1981). The principal source of iron is the RE system where phagocytosis of senescent red blood cells occurs. The liver, intestinal mucosal cells and the extravascular pool also contribute to a lesser degree to the plasma iron pool (Morgan, 1981). In times of increased demand for iron by the erythron, the requirement for transferrin iron is also increased (Bothwell et al. 1995). As a consequence, cells of iron procurement, particularly RE cells and hepatocytes, must have a mechanism for the release of iron to satisfy the

needs of the body. Most of the studies covering this aspect of iron metabolism have been conducted on RE cells and will be discussed in chapter 1.4.

The release of iron from hepatocytes has been investigated in various systems of hepatocytes (Baker et al, 1981; Baker et al, 1985; Beguin et al, 1989). It was found that the addition of apotransferrin and various iron chelators, such as citrate and desferrioxamine, to cells resulted in an increased rate of iron release. The release rate was also affected by experimental factors like incubation medium and source of iron (reviewed in Thorstensen and Romslo, 1990). The possibility of apotransferrin acting as a passive extracellular iron acceptor was suggested by Young and Aisen (1981) when no apotransferrin receptors were found to be present on hepatocytes.

1.3 IRON STORAGE - FERRITIN

1.3.1 Introduction

Ferritin is a specialized iron storage protein found in animals, plants, fungi and bacteria (Theil, 1987). The protein was first crystallized from horse spleen by Laufberger in 1935 and he suggested that ferritin played a role in iron storage (Crichton and Charloteaux-Wauters, 1987). It serves a significant function in the human body where it sequesters toxic free iron and provides an intracellular storage of iron in the soluble form (Harrison et al, 1977). Ferritin is also found in very small amounts in serum (Jacobs, 1985; Worwood, 1990), where its deter-

mination can be used to assess iron stores in patients. In the normal adult up to 1 gram of iron, depending on the individual's nutrition, is stored in the form of ferritin and haemosiderin (Bothwell et al, 1995). Ferritin is the soluble, mobile fraction found in the cytoplasm and haemosiderin is present within secondary lysosomes (Theil, 1987; Crichton and Charloteaux-Wauters, 1987). Normally, about two thirds of storage iron is in ferritin mainly found in parenchymal cells of the liver. The greatest source of iron in hepatic parenchymal cells is plasma transferrin. However, hepatocytes also take up haemopexin iron, haemoglobin-haptoglobin and ferritin iron (Hershko et al, 1972). The remaining one third of storage iron is in the form of haemosiderin within the RE cells of the liver, spleen, bone marrow and skeletal muscle (Bothwell et al, 1979; Powell and Halliday, 1982). The RE cells derive most of their iron by phagocytosing senescent red cells with removal of iron from haemoglobin. The proportion of iron in haemosiderin increases as the body iron stores increase, an essential feature of iron overload disorders. The resultant haemosiderin deposits can be used histologically to assess the size of body iron stores. Iron stored in all the abovementioned sites can be mobilized in the face of increased body needs, and then transported by plasma transferrin to sites of utilization. The mechanisms of iron removal from ferritin or its incorporation into apoferritin in vivo are poorly understood. The mechanism whereby iron regulates ferritin synthesis

has however received considerable interest in recent years. The induction of ferritin synthesis in a variety of tissues and cell lines by iron is now well established. Regulation at the level of transcription and translation has been described. Investigations of the molecular basis underlying the regulation of expression of ferritin by iron are currently progressing rapidly. Its significance in disorders of iron metabolism such as hereditary haemochromatosis remains to be explored.

This section describes the structural and functional characteristics of ferritin and the regulation of its biosynthesis with respect to iron.

1.3.2 Structure

Ferritin consists of a roughly spherical protein coat (apoferritin) enclosing a core of ferric-hydroxy-phosphate (Harrison et al, 1977). The protein shell (MW 45 kDa) can be isolated from ferritin solutions by density gradient centrifugation or by reduction of the ferric core and chelation of the ferrous ion (Chasteen and Theil, 1982; Crichton and Charloteaux-Wauters, 1987). The three dimensional structure of apoferritin has been solved by X-ray crystallography (Harrison, 1986). It is composed of 24 subunits packed in 4:3:2 symmmetry. Conservation of certain amino acid sequences is observed from amphibia to mammals (Theil, 1987). Each subunit consists of four long alpha helices (A to D) with a short helix E (Harrison et al, 1987). The subunits interact with each other by

hydrophilic and hydrophobic channels and salt bridges. Four subunits interact to form six hydrophobic channels and eight hydrophilic channels are formed by three subunits. It is thought that the hydrophilic channels are involved in the transport of iron in and out of the molecule (Theil, 1987).

The mineral core surrounded by the protein shell consists of hydrous ferric oxide containing some phosphate. Up to 4500 atoms of iron are held within each ferritin molecule (Mann et al. 1986). Iron is stored in the ferric state (Fe III) within ferritin but the ferrous iron (Fe II) enters ferritin via the eight hydrophilic channels where it is oxidized to Fe(III) (Ford et al. 1984). The process whereby ferritin iron is released in vivo is not yet clear. However, in vitro studies have shown that various ferric iron chelators and reducing agents (e.g. citrate, nitrilotriacetate, desferrioxamine, glutathione, ascorbic acid) are able to mediate iron release from ferritin (Crichton and Charloteaux-Wauters, 1987). Reductive mobilization of iron from ferritin by free radicals has also been shown (Thomas and Aust, 1986).

Haemosiderin is a water insoluble protein-iron complex, believed to be formed by the partial digestion of ferritin aggregates by lysosomal enzymes (Munro and Linder, 1978; Wixom et al. 1980). The iron within haemosiderin is very similar to that within ferritin (Richter, 1984) and can be mobilized during times of increased body needs, although it is not as accessible as ferritin (Bothwell et al. 1979).

1.3.3 Isoferritins

Multiple bands of ferritin can be observed by techniques such as electrophoresis in denaturing gels and isoelectric focusing. Arosio and co-workers (1978) proposed that ferritin molecules are composed of two subunit types designated H (heavy, heart) and L (light, liver). The H-subunit (MW 21 000 daltons) consists of 178 amino acids and the L-subunit (MW 19 000 daltons) has 174 amino acids (Drysdale, 1977; Arosio et al, 1978; Worwood 1990). The isoelectric points of the H- and L-subunits are 4,6 and 5,7 respectively (Bezkorovainy, 1989).

Heterogeneity of ferritin is observed in different tissues and is related to the presence of varying proportions of the two subunits. These different ratios between H and L give rise to a series of isoferritins (Arosio et al, 1978; Bomford et al, 1981) which differ in immunological and electrophoretic properties. H-rich isoferritins are more acidic whereas L-rich isoferritins are more basic in nature (Powell et al, 1975a). The isoferritin profile varies not only between tissues, but may also be altered in pathological conditions such as neoplasia and in cellular differentiation (Drysdale et al, 1977; Chou et al, 1986). H-rich isoferritins predominate in the heart, red blood cells, monocytes and lymphocytes and L-rich isoferritins are more abundant in the liver, spleen and placenta (Powell et al, 1975a; Worwood, 1986). The functional differences associated with H- and L-subunits remain unexplained, but it has been proposed that

the H-type ferritins turn over and take up and release iron faster than the L-type ferritins in vitro (Wagstaff et al. 1978; Bomford et al. 1981). In contrast, iso-ferritins rich in L-subunits appear to retain iron more firmly and are found in tissues capable of storing large amounts of iron. There is evidence to suggest that the synthesis of L-rich ferritins is increased upon iron loading of tissues (Bomford et al. 1981; Treffrey et al. 1984).

cDNAs encoding the H- and L-ferritin subunits and the corresponding genes have been isolated and characterized in various species and tissues (reviewed in Theil, 1987 and Drysdale, 1988). In humans, the H and L chains are derived from multigene families (Boyd et al. 1985; Jain et al. 1985). There are now about 15 H genes (Cragg et al. 1985; Costanzo et al. 1986) and at least 5 copies of the human L-gene (Drysdale, 1988). However, the H- and L-genes are only expressed on chromosomes 11 (11q13) and 19 (19q13ter), respectively (Worwood et al. 1985). It is generally thought that the other genes represent non-functional pseudogenes. It is also possible that the presence of ferritin genes on different chromosomes may protect cells against the loss of a functional gene as suggested by Jain and colleagues (1985).

1.3.4 Functions

The main function of ferritin is to store iron in a soluble non-toxic form (Harrison et al. 1977). Ferritin

can be classified into three functional types according to Theil (1987). These are : (i) Specialized cell ferritin which stores iron for use by other cells. Examples are the long term iron storage in hepatocytes, the recycling of iron to the erythron by erythrophagocytosing macrophages and the iron within embryonic red cells for rapid consumption; (ii) intracellular housekeeping ferritin which maintains iron reserves for cytochromes, nitrogenase, ribonucleotide reductase, haemoglobin and myoglobin and (iii) intracellular housekeeping stress ferritin involved in the detoxification of excess iron during iron overload. For example, iron and ferritin accumulate within macrophages in response to inflammation. The three forms of ferritin are therefore found in different cell types serving various functions and vary according to the physiological state of the cells in the body. Whether these various ferritins are structurally different, has not yet been elucidated. There has however been considerable interest in the transcriptional and translational control of ferritin synthesis by iron in various cell types. It appears that cells specialized for iron storage and provision of iron for use by other cells recruit existing ferritin messenger RNA (mRNA) for translation into ferritin, whereas cells that sequester iron to prevent toxicity or require iron for normal housekeeping functions exhibit changes in ferritin mRNA concentrations indicating control at a transcriptional level. This topic will be discussed in chapter 1.3.6.

Ferritin may subserve several other functions besides iron storage. It has been suggested that ferritin may play a role in transporting iron between Kupffer cells and hepatocytes within the liver (Sibille et al. 1988). Ferritin contains small amounts of copper and zinc and may therefore be involved in the storage of these elements (Price and Joshi, 1982). The exact function of serum ferritin is not known.

1.3.5 Ferritin as diagnostic tool

Small amounts of ferritin are normally present in plasma (Worwood, 1990). It is usually of low iron content (Worwood et al. 1976), consists almost exclusively of L-subunits and is partially glycosylated (Worwood, 1986). It is cleared much slower from plasma (half-life about 30 hours) (Worwood et al. 1982) than non-glycosylated tissue ferritins (half-life about 10 minutes) (Cragg et al. 1983).

The origin of serum ferritin is not clear. Its glycosylation suggests that it is synthesized by membrane-bound polysomes of phagocytes and secreted into the circulation (Bothwell et al. 1979). The main source of serum ferritin appears to be the RE system and the liver (Siimes and Dallman, 1974; Mack et al. 1981).

Normal concentrations of serum ferritin range from about 20 to 300 µg/liter depending on age, sex and nutritional iron status. Values below 12 µg/l are virtually diagnostic of iron deficiency whereas in

patients with hereditary haemochromatosis values may range from 1000 to 10 000 μg/l (Bothwell et al. 1995). Normally, the serum ferritin concentration is well correlated with the amount of storage iron in the body. It can therefore be used clinically to assess body iron stores (Lipschitz et al. 1974; Worwood, 1986). However, it must be remembered that raised serum ferritin levels may occur in pathological states such as infections (Baynes et al. 1986), inflammation and rheumatoid athritis (Baynes et al. 1987a), certain neoplastic diseases (Bezwoda et al. 1985) and in hepatocellular disease (Prieto et al. 1975). False results may be observed in severe iron overload due to extensive tissue damage, mainly of hepatocytes, causing ferritin to be released (Prieto et al. 1975) and ascorbate deficiency which lowers serum ferritin in iron overload (Roeser et al. 1980). All these limitations have to be taken into account when interpreting serum ferritin levels.

1.3.6 Ferritin gene regulation and synthesis

1.3.6.1 Introduction

The amount of chelatable intracellular iron is the most important regulator of ferritin synthesis. It has been shown repeatedly in many different cell types that increased cellular iron increases the synthesis of ferritin (reviewed in Munro and Linder, 1978; Theil, 1987 and Halliday et al. 1994).

Normally, the amount of protein synthesized by cells

can be altered by regulation of the mRNA populations. This can be achieved by changing the mRNA concentrations effected by transcription of the corresponding genes or by altering the stability of the mRNA. Alternatively, the regulation can be modulated by varying the utilization of mRNA (by storage or translational efficiency) (Theil, 1987). For iron-induced ferritin biosynthesis, the regulation primarily takes place at a translational level without changing the mRNA levels (Zahringer et al, 1976a; Aziz and Munro, 1986; Rogers and Munro, 1987). Two types of post-transcriptional regulation are involved : (i) recruitment of stored ferritin mRNA with no change in the amount of translatable ferritin mRNA (Zahringer et al, 1976a) or hybridizable ferritin mRNA (Aziz and Munro, 1986; Rogers and Munro, 1987) and (ii) competitive translation of derepressed ferritin mRNA (Shull and Theil, 1982). Translational control of protein synthesis is unusual since most cells make use of the transcriptional mechanism. As alluded to in chapter 1.3.4, translational control seems to feature more in cells specialized for iron storage where the stored ferritin mRNA can be rapidly recruited for translation into ferritin. In recent years sequences within ferritin mRNA involved in the iron-dependent regulation of ferritin biosynthesis, as well as cytoplasmic factors that bind to these sequences, have been characterized.

Control of ferritin synthesis by iron can also occur at the level of transcription with changes observed in the

ferritin mRNA content (Cairo et al, 1985; White and Munro, 1988). This mode of regulation appears more important in cells which require iron for housekeeping functions and in cells involved in the detoxification of excess iron (Dickey et al, 1987; Theil, 1987). Here the stress of increased intracellular iron stimulates transcription to increase previously low ferritin mRNA concentrations. A preferential emphasis on the L-subunit has been noted in situations of high iron stress (White and Munro, 1988) which is in agreement with the finding that L-rich ferritins can store large amounts of iron (Bomford et al, 1981; Treffrey et al, 1984).

Numerous other agents are capable of inducing the expression of the ferritin gene. It has been shown that thyrotropin (Colucci-D'Amato et al, 1989; Chazenbalk et al, 1990), triiodothyronine (Iwasu et al, 1990), tumor necrosis factor (Torti et al, 1988) and insulin (Yokomori et al, 1991) can enhance transcription.

The site of ferritin synthesis within cells depends on whether ferritin is exported or kept within the cell. Ferritin for export (only 20%) is made on membrane-bound polysomes, whereas ferritin for intracellular use is synthesized predominantly on free ribosomes in the cytoplasm (Hicks et al, 1969; Lee and Richter, 1977).

1.3.6.2 Translational control

The suggestion that ferritin synthesis is regulated at a post-transcriptional level came from initial studies making use of transcription inhibitors such as Actinomycin

D and cordycepin. The stimulatory effect of iron on ferritin synthesis was not suppressed by these agents (Drysdale and Munro, 1965; Zahringer et al, 1976a). Under physiological conditions, the majority of ferritin mRNA exists in a masked form in the cytoplasm as messenger ribonucleoprotein (mRNP) particles. Zahringer and co-workers (1976b) observed that 15% of total rat liver poly(A)-containing RNA was present in the post-microsomal supernatant. This fraction was highly enriched with ferritin mRNA accounting for 40-50% of the total ferritin mRNA present in the cytoplasm. It was subsequently found by the same authors that upon iron administration, the pool of inactive ferritin mRNA from the mRNPs was released from storage and entered polyribosomes for active translation (Zahringer et al, 1976a). This recruitment of stored message without changes in the concentration of ferritin mRNA has been confirmed by hybridization studies with cDNA probes in in vitro experiments on cultured rat hepatoma cells (Rogers and Munro, 1987) and in animal studies (Aziz and Munro, 1986). In addition, Rogers and Munro (1987) demonstrated that when iron was added as hemin, ferritin mRNA was also translocated to the polysome fraction. The addition of the iron chelator desferrioxamine, which crosses cell membranes, inactivated the response to hemin. This indicates that the amount of chelatable iron within cells controls ferritin mRNA translation. However, Mattia and co-workers (1989) reported that prolonged incubation of K562 human erythroleukaemia

cells with hemin results in increased ferritin mRNA levels which are not quenched by desferrioxamine, indicating the presence of a non-chelatable component. Alternatively, stabilization of the message by iron was proposed.

Competitive translation also plays a role in translational activation. Derepressed ferritin mRNA (by iron) is a strong competitor for translation initiation (Schaefer and Theil, 1981). This and the fact that ferritin mRNA has an extremely high translational efficiency (Shull and Theil, 1982) allows cells specialized for iron storage to respond efficiently to iron.

As previously mentioned, the ferritin subunit mRNAs exist within the cell as a pool of translationally inactive mRNP. The molecular mechanisms whereby iron induces translational activation of ferritin synthesis is closely coordinated with the iron-dependent modulation of transferrin receptor mRNA. Both will therefore be discussed here. Excess iron results in increased translation of ferritin mRNA and a decrease in stability of transferrin receptor mRNA. These regulatory events are mediated by the presence of a highly conserved 28-base stem looped structure called the iron-responsive element (IRE) contained in the 5'-untranslated region of both H and L ferritin mRNA (Aziz and Munro, 1987; Hentze et al. 1987). Five variations of these IREs are also found in the 3'-untranslated region of transferrin receptor mRNA (Casey et al. 1988). A 95 kDA cytoplasmic regulatory protein termed by various authors as the iron-responsive element binding

protein (IRE-BP); iron regulatory factor (IRF); ferritin repressor protein (FRP) or P-90 (reviewed in Klausner et al. 1993), interacts with the IREs from both ferritin and transferrin receptor transcripts (Leibold and Munro, 1988; Rouault et al. 1988; Mullner et al. 1989). More recently, a consensus was reached to name the cytoplasmic binding protein (formerly IRE-BP) the iron regulatory protein (IRP). The mechanism by which the IRP mediates cellular iron homeostasis has emerged from the observation that the human IRP is both structurally similar to mitochondrial aconitase from porcine heart and yeast (Hentze and Argos, 1991; Rouault et al. 1991) and directly possesses aconitase activity (Kaptain et al. 1991). Aconitase is an iron-sulphur (Fe-S) protein that catalyzes the conversion of citrate to isocitrate within the Krebs cycle. Purified aconitase exists in an active enzymatic [4Fe-4S] state and in an inactive [3Fe-4S] state (Beinert, 1990). Like aconitase, the IRP also possesses a dynamic Fe-S centre that can interconvert between two forms. The binding affinities of these forms for the IREs are determined by the iron status of the cell which appears to set the redox state of the IRP (Haile et al. 1989; Hentze et al. 1989b). The interaction of the IRP with the IRE has been proposed by Harford and colleagues (1994) to occur as follows : When iron is in excess, the IRP is in its [4Fe-4S] state which has high aconitase activity but low affinity for the IREs, resulting in increased translation of ferritin mRNA and decreased stability of the transferrin receptor mRNA.

Therefore more iron is deposited into ferritin for storage and less iron is taken up by the cell. Conversely, when cellular iron becomes limiting, the IRP exists in its [3Fe-4S] state with negligible aconitase activity but high affinity for the IREs, resulting in reduced ferritin synthesis for iron storage and increased stability of transferrin receptor mRNA for more iron uptake. It is interesting that the genes for human cytosolic aconitase and the human IRP are both localized to chromosome 9 (Rouault et al, 1990). In fact it is now believed to be one and the same protein with two functions. The finding of IRE-like structures in mRNAs encoding 5-aminolevulinate synthase (Cox et al, 1991; Dandekar et al, 1991) and mitochondrial aconitase (Dandekar et al, 1991; Kaptain et al, 1991) suggests that iron may also play an important regulatory role in the metabolic pathways of haem biosynthesis and the citric acid cycle. Evidence has recently been provided of the existence of a second IRP termed IRP2 which does not possess aconitase activity and differs partially (from the well-characterized IRP) in the regulation, distribution and binding specificity (Guo et al, 1995; Kuhn et al, 1995).

1.3.6.3 Transcriptional control

Despite the dominant effect of iron on ferritin translation, there is evidence that ferritin expression is regulated by transcription (or mRNA stability) during growth, differentiation and iron overload. Developmentally associated changes in ferritin mRNA levels have been

demonstrated in human promyelocytic leukaemia cells (HL-60) and in mouse Friend erythroleukaemia cells. A substantial increase in the ratio of H- to L-subunit mRNA was shown to occur during the in vitro differentiation of HL-60 cells to neutrophils or macrophages (Chou et al, 1986) as well as during erythroid differentiation of Friend erythroleukaemia cells (Beaumont et al, 1987). Although the factors responsible for these changes during the differentiation process are not known, it is possible that variations in cellular requirements for iron may play a role. The influence of iron on the synthesis of housekeeping ferritin has been studied in adult frog red cells (Dickey et al, 1987). They showed that iron induces a change in the relative amounts of ferritin subunit mRNA in order to sequester any excess iron, by a process of transcription or altered stability. However, in embryonic red cells specialized for iron storage the existing high mRNA levels are rapidly translated into ferritin. Further evidence for enhanced transcription of ferritin genes by iron loading has been provided by studies on HeLa cells. Cairo and co-workers (1985) demonstrated that upon incubation of HeLa cells with iron, H- and L-mRNA levels increased suggesting transcriptional control. However, the increase in ferritin mRNA levels could not account for the large increase in the actual ferritin level. Regulation at a post-transcriptional level was proposed to be involved as well. This coordination of both levels of regulation was further demonstrated by White and Munro (1988). It was

shown in rat liver that iron induced transcription of H and L genes in addition to a translational shift of both subunit mRNAs to polysomes; the latter mechanism allowing the cell to respond quickly to iron. It was also observed that the L-subunit was preferentially stimulated over the H-subunit, confirming previous findings that L-rich isoferritins are more suited for iron storage (Bomford et al. 1981).

1.4 NORMAL RETICULOENDOTHELIAL IRON METABOLISM

1.4.1 Introduction

The reticuloendothelial system (RES), also known as the mononuclear phagocyte system, consists of a diverse group of highly phagocytic cells. All cells of this system originate from precursor cells in the bone marrow giving rise to peripheral blood monocytes (Cline et al. 1978). The blood monocytes form 4 to 8% of the total white cell count and have a circulating half-life of about 32 hours. Monocytes differentiate into histiocytes or resident macrophages within connective tissues of organs. These include hepatic Kupffer cells, perivascular macrophages of sinusoids of the spleen, lymph nodes and bone marrow, alveolar macrophages, pleural and peritoneal macrophages, microglial cells in the nervous system, epidermal Langerhans cells and osteoclasts of bone (Cline et al. 1978). Reticulum cells not only synthesize reticulin fibres in tissues with a supporting framework of reticular fibres, but also exhibit considerable phagocytic activity.

Due to their phagocytic properties, the macrophages are active in the defence against injurious agents. They have a fundamental role in the immune response and secrete many biologically active products (Nathan, 1987; Auger, 1989). The RE cells, particularly of the liver, spleen and bone marrow, play a central role in iron metabolism. They are responsible for the destruction of effete and senescent red blood cells and for recycling the iron released for synthesis of haemoglobin in the bone marrow (Lynch et al. 1974; Deiss, 1983). In addition, the RES serves as one of the major iron storage sites of the body providing an iron reserve which can be mobilized and protecting the organism against the damaging effects of iron (Finch and Huebers, 1982). Furthermore, the RES plays a role in the regulation of iron absorption via the amount of iron stored (Bezwoda et al. 1979; Rosenmund et al. 1980). The precise regulatory mechanism by which this process is effected is unknown.

In the sections that follow, iron entry into RE cells, intracellular events and iron release by RE cells will be discussed.

1.4.2 Iron uptake by RE cells

The RES derives most of its iron from haemoglobin of broken down red cells. Of the 30 mg of iron exchanged daily with the RES, two thirds are derived from erythrocytes and the remainder from ineffective erythropoiesis (Cook et al. 1970). Phagocytosis of senescent or damaged

red cells occurs predominantly in the RE cells of the spleen, liver and bone marrow (Deiss, 1983). To study iron uptake experimentally, red cells labelled with radioiron can be damaged by physical, chemical or immunological means and then infused. The splenic cords of the red pulp appear to be the main site of phagocytosis of effete red blood cells (Rosse, 1987). It is still not clear whether the destruction is purely a mechanical process or whether immunological recognition may play a role. Phagocytosis of red cells has also been observed in the liver and bone marrow under different conditions (Hughes Jones and Cheney, 1961; Frank et al, 1972). Deiss and Cartwright (1970) documented that RE cells may directly receive small amounts of ferritin from within red blood cells. Haemoglobin bound to haptoglobin or hemopexin, free haemoglobin and hemin are not removed by RE cells, but by hepatocytes and kidney cells (Hershko et al, 1972).

A small amount of iron may also be delivered to RE cells by the iron transport protein, transferrin. Several studies have reported transferrin-bound iron uptake by cultured macrophages (Summers and Jacobs, 1976; Wyllie, 1977; Sizemore and Bassett, 1984; Baynes et al, 1987b). The number of transferrin receptors on monocyte-macrophages varies substantially in different situations. Transferrin receptor expression is absent or decreased on human monocytes and on resident (quiescent) and activated (fully cytolytic) macrophages. However, the expression is increased on responsive macrophages (cells that become

cytolytic in response to an appropriate signal) and on cultured macrophages (Andreesen et al. 1983 & 1984; Adams and Hamilton, 1984; Hirata et al. 1986; Testa et al. 1989). With regard to iron and transferrin receptor expression in RE cells, an interesting study by Testa and co-workers (1989) revealed that iron salts induce an upregulation of both transferrin receptor RNA and transferrin receptors in human cultured maturing macrophages. This was supported by a recent study demonstrating that iron-transferrin enhances transferrin receptor expression in nonactivated cultured monocytes (Byrd and Horwitz, 1993). These findings are in sharp contrast to the effect observed in most other cells types: excess iron leading to a downregulation of transferrin receptor expression (discussed in chapters 1.2.5.3 and 1.3.6.2). It has been suggested that the increase in transferrin receptors allows macrophages to store large amounts of iron.

In most studies it has only been speculated that transferrin iron is taken up by cultured macrophages by a process of receptor mediated endocytosis. Baynes and co-workers (1987b) studied the effect of various metabolic inhibitors on receptor mediated endocytosis and concluded that an endocytic pathway similar to that in erythroid precursors may be present in human cultured blood monocytes. The biological significance of these observations and the role of the transferrin receptor is not clear since RE cells derive most of their iron from degraded haemoglobin.

The iron-binding glycoprotein, lactoferrin has been suggested to be involved in the delivery of iron to RE cells. Uptake of iron from lactoferrin has been demonstrated in mouse peritoneal macrophages (van Snick et al, 1977) and in human monocytes (Birgens et al, 1988). Lactoferrin receptors have also been identified on monocytes (Birgens et al, 1983).

1.4.3 Intracellular iron metabolism

Erythrocytes that have been engulfed by phagocytosis are degraded by lysosomal enzymes which are released by fusion of the phagocytic vesicles with the lysosomes (Lynch et al, 1974). After globin is split from methaemoglobin, iron is released from the haem component by the action of microsomal haem oxygenase (Tenhunen et al, 1972) with the formation of carbon monoxide and biliverdin. Haem oxygenase activity is low in monocytes but is induced by uptake of haemoglobin (Schacter, 1988).

The iron from haemoglobin catabolism may either return to the plasma or become stored as ferritin or haemosiderin. Although the major portion of this iron is released to transferrin (Noyes et al, 1960), many factors such as body iron stores, erythropoiesis, inflammation and ascorbic acid deficiency may affect the balance between the two pathways. The passage of iron through RE cells has been characterized in dogs (Fillet et al, 1974) and in humans (Fillet et al, 1989) using radiolabelled heat damaged red cells. From these studies it appears that iron

released from haem passes through a labile intracellular iron pool from which it either returns to the plasma or exchanges with the slow storage pool. Iron release from RE cells will be discussed in chapter 1.4.4.

The slow storage pool represents ferritin and haemosiderin. As mentioned previously (chapter 1.3.4), macrophages may contain three types of ferritin namely specialized cell ferritin for use by other cells, housekeeping ferritin for intracellular purposes and housekeeping stress ferritin for situations of iron overload. Monocytes contain ferritin with a relatively high H-subunit content (Powell et al. 1975a; Jones et al. 1983). However, their in vitro maturation to macrophages is associated with an increase in L-rich isoferritins characteristic of iron storage organs such as liver and spleen (Worrall and Worwood, 1991). Accumulation of L-rich isoferritins has also been observed in macrophages after erythrophagocytosis (Harrison, 1986; Raha-Chowdhury et al. 1993) or by incubation with iron salts (Worrall and Worwood, 1991). It is not known whether this increase was caused by transcriptional or translational mechanisms. A more detailed study conducted by Testa and co-workers (1989) showed that expression of H and L ferritin chains at RNA and protein levels was considerably higher in cultured macrophages than in fresh monocytes. This indicates either enhanced transcription of the ferritin genes or regulation at a translational level. Upon the addition of iron as ferric ammonium citrate, an increase

in both H- and L-ferritin was associated with only a moderate rise in the corresponding RNA components, suggesting regulation at a translational level. Byrd and Horwitz (1993) similarly showed that incubation of cultured macrophages with transferrin-bound iron results in an up-regulation of the intracellular ferritin content. It appears that iron regulation of ferritin synthesis in monocyte-macrophages may occur at either transcriptional or translational levels or both depending on the degree of differentiation or the cell's iron status or its particular function at any one time.

1.4.4 Iron release by RE cells

The RES serves as the major source of iron for the plasma iron pool (Morgan, 1981). Transferrin is responsible for transporting this iron to the erythron for haemoglobin synthesis. RE cells release iron in proportion to body needs. In iron deficiency, the iron from haemoglobin catabolism returns to the plasma with very little being stored (Bentley et al. 1979). The level of erythropoiesis also affects RE iron release. For example stored iron is rapidly mobilized in situations of acute bleeding. However, in two clinical situations this pattern does not apply. In the anaemia of chronic disorders, low plasma iron and transferrin saturation are coupled with greater than normal levels of storage iron in RE cells and mucosal cells (Roeser et al. 1980), suggesting a failure of mononuclear cells to release iron despite a significant

concentration gradient. In contrast, in idiopathic haemochromatosis plasma transferrin and parenchymal cells are markedly iron overloaded with very little iron in the RE cells (Brink et al, 1976), possibly indicating release by these cells against a concentration gradient. From these observations, the release of iron from RE cells may be effected by an active process. The possibility that apotransferrin receptors within the membrane of RE cells may play a role in iron release has been investigated. A study by Baynes and co-workers (1987b) on cultured human blood monocytes provided no evidence of apotransferrin receptors indicating that iron release from the RE cells does not occur via this pathway.

RE iron kinetics in dogs showed that radioiron is released from RE cells in a biphasic manner after the infusion of labelled heat damaged red cells. It was shown that there was an initial early release phase (from haem catabolism) with a half-time of 34 minutes and a late release phase (release from RE stores) with a half-time of 7 days (Fillet et al, 1974). A subsequent study investigating RE iron kinetics in vivo in humans was presented by Fillet and co-workers, 1989. It was shown that after a lag period of about 40 minutes, two thirds of the freed radioiron from haemoglobin were returned to the plasma with a half-time of 33 minutes and one-third was incorporated into RE stores and released at a slower rate (half-time of 6 days).

Iron may be released in the form of ferritin by RE cells. Brock and co-workers (1984) showed that a small amount of ferritin was released from mouse peritoneal macrophages after their incubation with radiolabelled transferrin-antitransferrin immune complexes. Similarly, isoferritins were detected in the iron-containing medium of peripheral blood mononuclear cells (Worwood et al, 1984). Using a haemolytic plaque forming assay, de Sousa and colleagues (1982) provided further evidence of ferritin secretion from mononuclear phagocytes. A recent in vitro study investigating the molecular form in which iron is released from human monocytes and monocyte derived macrophages, showed that 25 to 30% of released iron appeared to be present as low molecular weight iron compounds in the cell culture supernatants. Ferritin-bound iron could not be detected (Moura et al, 1995).

The release of iron from RE cells also appears to be dependent upon the availability of caeruloplasmin. Caeruloplasmin acts as a ferroxidase catalyzing the conversion of ferrous iron to the ferric state, the latter being normally bound to transferrin. Caeruloplasmin may therefore facilitate the mobilization of iron from RE cells by this mechanism (Frieden and Osaki, 1974).

1.5 HLA-LINKED HAEMOCHROMATOSIS

1.5.1 Introduction

The term "haemochromatosis" was first introduced by von Recklinghausen in 1889. The pathological term can be defined as the presence of excessive amounts of iron in parenchymal cells leading eventually to cellular damage and functional impairment of organs, while the term "haemosiderosis" is reserved for tissue iron overload without evidence of cellular damage (Bothwell et al. 1995). The causes of haemochromatosis are multiple. The most common and the most severe iron overload is found in the genetic disorder; it has been designated idiopathic, primary, genetic or hereditary haemochromatosis. HLA-linked haemochromatosis may be the most appropriate term since HLA association defines the disease. The other causes of haemochromatosis can collectively be called secondary haemochromatosis. These include causes secondary to anaemia and ineffective erythropoiesis (e.g. thalassaemia and sideroblastic anaemia, usually compounded by multiple transfusions), to liver disease (e.g. alcoholic cirrhosis and following portocaval anastomosis) and to high oral iron intake in African iron overload (Powell et al. 1980).

This dissertation is concerned with HLA-linked haemochromatosis. In the sections that follow the genetics, pathogenesis, clinical features and diagnosis of the disease will be briefly discussed. The sites of possible abnormalities of hereditary haemochromatosis are also dealt with here.

1.5.2 Genetics

HLA-linked haemochromatosis occurs as a result of an inborn error of metabolism in which dietary iron absorption is inappropriately increased in relation to body iron stores (Bothwell et al, 1995). The disease is inherited as an autosomal recessive trait. Sheldon (1935) first suggested that this disease was inherited, but it was only found much later that the phenotypic expression of haemochromatosis was linked with HLA haplotypes (Simon et al, 1975 & 1977). The HLA antigen A3 occurs in about 70% of individuals with this condition, while only 28% of the general European population are HLA-A3 (Simon et al, 1980). In addition, HLA-B7 and HLA-B14 (Walters et al, 1975a; Simon et al, 1976) and HLA-A2 and HLA-A11 (Summers et al, 1990) have also been associated to a lesser extent with hereditary haemochromatosis.

The gene for hereditary haemochromatosis has not yet been identified but is known to lie close to the HLA-A locus on the short arm of chromosome 6 (6p) (Edwards et al, 1980a; Powell et al, 1994). Recent studies making use of recombination mapping have confirmed that the gene is in fact very closely linked to HLA-A2 (Gasparini et al, 1993; Jazwinska et al, 1993). cDNAs which may encode the haemochromatosis gene have been identified (El Kahloun et al, 1993). The gene frequency for hereditary haemochromatosis is about 0,05 in most Caucasian populations corresponding to a heterozygote frequency of 10%. Approximately 3 in every 1000 individuals are homozygous for

haemochromatosis (Simon et al, 1977; Cartwright et al, 1979). Full clinical expression of the disease is most commonly seen in individuals homozygous for the gene. Heterozygotes have less pronounced metabolic aberrations and often only have mild biochemical abnormalities (Simon et al, 1980).

1.5.3 Pathogenesis and clinical manifestations

Normal total body iron is about 4 g (Bothwell et al, 1979). In patients with HLA-linked haemochromatosis the total body iron content is 15 to 40 g. However, the iron stores are increased between 20 to 50 times the normal as the surplus iron is held within the storage compounds (Bothwell et al, 1979). The iron stores accumulate over a number of years and with an excessive absorption amounting to 4 mg per day or more, the disease usually becomes clinically manifest at 40-60 years of age. Males have a 10-fold greater disease incidence than females. This can be attributed, in part, to iron losses afforded by menstruation, pregnancy and lactation and to a low dietary intake in females (Bothwell et al, 1995). Since the phenotypic expression of the gene is likely to depend on the amount of available iron in the diet, a high dietary iron intake and excessive alcohol consumption may accelerate the accumulation of iron (Bothwell et al, 1995).

The excess iron is deposited in parenchymal cells mainly in the form of haemosiderin. The organs and tissues most commonly affected are the liver, pancreas, heart,

endocrine organs, skin and joint linings. An integral part of the disease is the insignificant involvement of the RES and the intestinal mucosal cells (Valberg et al, 1975; Brink et al, 1976; McClaren, 1989). This contrasts with transfusion iron overload where the iron is deposited largely in the mononuclear phagocyte system.

The principal manifestations of hereditary haemochromatosis include cirrhosis, cardiomyopathy, diabetes mellitus, hypogonadism, skin pigmentation and athritis (Bothwell et al, 1995). It has been proposed that iron causes tissue injury by the formation of free radicals such as hydroxyl radicals, which then cause lipid peroxidation of subcellular organelles (Bacon and Britton, 1989). A recent study has suggested that increased plasma levels of thiobarbituric acid-reactants found in patients with hereditary haemochromatosis could be the result of increased peroxidation (Young et al, 1994). The disruption of iron-laden lysosomes leading to the release of hydrolytic enzymes (Selden et al, 1980), damage to DNA, reduced cellular levels of ATP and impaired cellular calcium homeostasis may all contribute to cellular injury in iron overload (reviewed in Britton et al, 1994).

The primary treatment of HLA-linked haemochromatosis is to remove the excess iron by repeated phlebotomy (Bothwell et al, 1979). Initially, weekly or even twice weekly venesections of 500 to 600 ml of blood for about 1-3 years is required. Thereafter, maintenance venesection every 3 to 4 months is required to counteract further accumulation of iron (Bothwell et al, 1995).

1.5.4 Diagnostic methods

Once hereditary haemochromatosis is suspected, the body iron status can be assessed by various biochemical tests. The combined measurements of the (1) serum iron (SI) concentration (2) percent transferrin saturation and (3) serum ferritin (SF) levels provide a reliable screening test for the disease. The SI level and percent saturation of transferrin are elevated early in the course of the disease, even before the body iron content is increased (Bassett et al, 1984). The SI concentration is usually above 200 μg/dL (normal 60 to 150 μg/dL) in the later stages. The SI levels may however be normal in young male patients and in some females with the disease (Edwards et al, 1980b) and may be increased with iron medication (Bothwell et al, 1995). The percentage saturation of transferrin (serum iron divided by total iron binding capacity) is usually a reliable indicator of hereditary haemochromatosis. A saturation above 62% has been found in 92% of individuals homozygous for the disease (Dadone et al, 1982). A transferrin saturation greater than 70% is virtually diagnostic of iron overload. The transferrin saturation may however be decreased by the concomitant presence of infection or a hepatoma (Bothwell et al, 1995). To eliminate false positives and false negatives the saturation must be measured on more than one occasion, preferably fasting.

The SF level is proportional to body iron stores and is therefore a useful indicator of the iron storage

compartment (Lipschitz et al, 1974; Worwood, 1986). SF may be elevated out of proportion in certain clinical conditions such as infection, malignancy and hepatic necrosis (discussed in chapter 1.3.5) which makes it a poor indicator of iron overload on its own.

The definitive test of HLA-linked haemochromatosis is liver biopsy. It permits histological staining for iron, measurement of iron concentration and assessment of the extent of liver damage. The hepatic iron index (liver iron concentration divided by age) with values greater than 1.9 is useful in the diagnosis of the disease (Bassett et al, 1986). Newer noninvasive tests for measuring body iron stores are being developed and include computed tomography, magnetic resonance imaging and magnetic susceptibility measurement (Brittenham, 1988), but these are not suitable for routine use.

Screening of siblings of known homozygotes by HLA typing together with studies of their iron status is important in the early diagnosis of hereditary haemochromatosis (Haddy et al, 1988).

1.5.5 Nature of the metabolic defect

1.5.5.1 Introduction

The underlying metabolic defect which results in iron overload in HLA-linked haemochromatosis remains to be elucidated. Numerous proposals have been made as to the possible sites at which an abnormality may be found. These sites include the intestine, liver and RES. An abnormality

in transferrin or its receptor and ferritin has also been suggested. A complete discussion of each site is beyond the scope of this dissertation and only the most important aspects are highlighted. More emphasis is placed on RE iron metabolism in haemochromatosis.

The Intestine. The reason for the increased iron absorption inappropriate to body needs remains unknown. An abnormality in the mucosal epithelium of the upper small intestine has been suggested by numerous investigators. From in vitro studies Cox and Peters (1978) proposed an increased avidity for iron by mucosal cells. Evidence to suggest that the mucosal storage pool may be impaired has been provided by studies showing that the ferritin content is reduced in these cells (Crosby, 1963; Fracanzani et al, 1989). Other studies have suggested that the mucosal iron uptake and transfer of mucosal iron to the circulation are increased in haemochromatosis (Powell et al, 1970; Marx, 1979). Recent work by Pietrangelo and co-workers (1992 & 1993) demonstrated an increase in the steady state mRNA for the transferrin receptor in duodenal mucosa, whereas for ferritin both mRNA and protein levels were reduced. It was suggested that a reduced iron pool may have led to this effect. Furthermore, it has been shown that the coordinate regulation by iron of ferritin synthesis and transferrin receptor expression (IRP-IRE mechanism) appears to be intact in haemochromatosis (Pietrangelo 1993). Of importance here, is the in vivo investigation of mucosal iron transport kinetics by McClaren and co-workers

(1991) in which it was demonstrated that the increased iron absorption is mediated mainly by an increase in the rate constant for transfer of iron from the mucosa to plasma rather than an abnormality in the regulation of muc

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The Liver. From studies conducted by Pollycove et al (1971) and Batey et al (1978) on treated patients with haemochromatosis, it has been suggested that the hepatic parenchymal cells have an increased affinity for iron. This can not be so since iron stores are easily mobilized following phlebotomy of haemochromatosis patients (Bothwell et al, 1979). The hepatocyte iron uptake by various pathways has been identified and discussed in chapter 1.2.6.1. A study by Sciot and co-workers (1987)

using immunohistochemical staining of liver biopsies showed that in untreated haemochromatotic patients the transferrin receptor expression was absent on hepatocytes. These findings are in keeping with the known effect of iron on transferrin receptor expression [see chapter 1.2.5.3]. In the absence of transferrin receptors the liver however continues to accumulate more iron. Other pathways for iron uptake may become operative. The uptake of non-transferrin-bound iron is vitally important in haemochromatosis where up to 30% of serum iron is not bound to transferrin (Batey et al, 1980). Liver ferritin does not appear to be defective in hereditary haemochromatosis. Although an abnormal pattern of liver isoferritins is observed in untreated patients (Powell et al, 1974), the pattern is also seen in other types of iron storage disease (Powell et al, 1975b). Furthermore, with depletion of iron the abnormal pattern returns to normal.

The RES. Possible defects in iron metabolism in RE cells of patients with haemochromatosis are discussed below.

1.5.5.2 RE iron metabolism

In hereditary haemochromatosis iron deposition in RE cells is inappropriately low compared to the size of parenchymal iron stores (Ross et al, 1975; Valberg et al, 1975; Brink et al, 1976). The transferrin saturation is increased even before the body has accumulated significant amounts of iron (Bothwell et al, 1995). This suggests a defect in RE iron uptake or storage in the disease. Three

aspects which may explain the abnormal behaviour of these cells have been extensively investigated and include iron uptake, iron storage and iron release.

Iron Uptake. Normally, iron absorption is inversely related to RE iron stores (Charlton et al, 1977; Bezwoda et al, 1979; Rosenmund et al, 1980). An impaired RE iron uptake may therefore result in an abnormally increased level of iron absorption. Although RE cells derive most of their iron from effete red blood cells, a small amount can be obtained from circulating transferrin [discussed in chapter 1.4.2). Several in vitro studies have investigated the latter pathway. Studies on peripheral blood monocytes have shown no difference in transferrin iron uptake between treated haemochromatotic patients and normal control subjects (Jacobs and Summers, 1981; Sizemore and Bassett, 1984). The work by McClaren and co-workers (1979) does not support this observation. They showed that the mean iron uptake from 100% saturated transferrin by monocytes from treated haemochromatotic patients was significantly lower than that demonstrated in normal monocytes (uptake of 3,16 ± 2,79 pmol/10^6 cells in treated patients compared with 12,32 ± 7,6 pmol/10^6 cells in normals; $p < 0,02$). It was concluded that a defect in iron uptake may account for the paucity of iron in RE cells. Interestingly, Sizemore and Bassett (1984) demonstrated a significant reduction of transferrin iron uptake by monocytes from iron-loaded haemochromatotic patients (uptake of 0,96 ± 0,30 fg/cell for iron-loaded patients

compared with 1,99 ± 0,87 fg/cell for normal subjects; $p < 0,01$). The authors suggested that the effect observed in monocytes from iron-loaded patients was merely a homeostatic mechanism of cellular iron metabolism.

The possibility of a defect in transferrin receptors has been raised. An extensive study on cultured human blood monocytes was conducted by Baynes and colleagues (1989). Scatchard analysis of binding curves failed to demonstrate any significant difference in the number or binding affinities of transferrin receptors on cultured macrophages from haemochromatotic patients and normal subjects. The results are in agreement with the findings of Ward and co-workers (1984a). In this study no differences in the properties of transferrin receptors on cultured fibroblasts or lymphocytes between patients with haemochromatosis and normal subjects were found. In contrast to the above, transferrin receptor expression has been found to be increased in peripheral blood monocytes from patients with haemochromatosis (Bjorn-Rasmussen et al, 1985). The biological significance of enhanced receptor expression is unclear, since RE cells in haemochromatosis accumulate very little iron.

Of interest is a report by Testa and co-workers (1989) that iron up-regulates transferrin receptor mRNA and transferrin receptors in normal cultured macrophages, suggesting an increased ability of these cells to take up more iron for storage. It is not known whether haemochromatotic mononuclear phagocytes are subject to the same mode of regulation.

It is important to remember that the uptake of transferrin iron by RE cells *in vivo* is relatively insignificant compared to the amount of iron obtained from erythro-phagocytosis. Therefore, studies that overemphasize the former pathway as playing a major role in the possible pathogenesis of hereditary haemochromatosis must be viewed with caution.

Iron Storage. An inability of RE cells to synthesize ferritin and store iron might also explain the scarcity of RE iron stores, the increased absorption and excess deposition of iron in parenchymal cells. Although Green and colleagues (1978) reported that monocyte ferritin content is disproportionately low in haemochromatosis, most studies do not support the hypothesis that ferritin synthesis is abnormal in RE cells. In a study comparing 7 treated haemochromatotic patients and 24 normal subjects, no difference between the two groups in either ferritin synthesis or the amount of iron incorporated into ferritin was observed (Jacobs and Summers, 1981). This was confirmed by a study demonstrating that monocyte ferritin synthesis, after induction with ferric ammonium citrate or iron dextran, did not differ significantly between treated patients and control subjects (Bassett *et al.* 1982). A study conducted by Worrall and Worwood (1991) on the immunological properties of ferritin showed that monocyte H- and L-type ferritins increased to the same extent (after iron induction) in patients with haemochromatosis and normal subjects. With maturation in culture to macro-

phages, the ratio of L- to H-type ferritin subunits increased similarly in both study groups. From the above observations it appears unlikely for there to be an abnormality in ferritin synthesis in haemochromatotic RE cells.

However, in an interesting report by Gordeuk and co-workers (1992) cultured monocytes from haemochromatosis patients released decreased amounts of tumor necrosis factor-α (TNF-α) compared with normal cells. TNF-α induces the expression of ferritin-heavy chain mRNA (Torti et al, 1988) and reduces the amount of iron released by peritoneal macrophages (Alvarez-Hernandez et al, 1989). The author therefore suggested that a reduced amount of TNF-α from haemochromatotic monocytes may result in inhibition of ferritin synthesis and enhanced release of iron from these cells. It should be noted that the gene for TNF-α is located close to the HLA locus on chromosome 6 (Spies et al, 1986), the same chromosome on which the gene for hereditary haemochromatosis is found.

It is well known that iron greatly influences translation of ferritin mRNA effected by binding of the IRP to IREs [discussed in chapter 1.3.6.2]. It has been postulated that a mutation in the IRP may account for the abnormal iron metabolism in haemochromatotic mononuclear phagocytes (Kuhn, 1991). It does not appear likely since the IRP is coded on chromosome 9 (Hentze et al, 1989a) rather than chromosome 6.

Iron Release. The paucity of iron in RE cells may relate to enhanced release of iron. Very little is known about this aspect in haemochromatotic cells. Flanagan and co-workers (1989) documented an increase in the release of ferritin from monocytes derived from untreated and treated haemochromatotic patients. The iron content of the ferritin was however not determined. The observations of this study await confirmation.

Iron release from normal and haemochromatotic macrophages was found not to increased when the cells were incubated in the presence of an extracellular-acting hydrophillic chelator (Baynes *et al.* 1989). Furthermore, Baynes and co-workers (1989) showed that apotransferrin does not appear to be essential for iron release.

The first *in vivo* study in humans to investigate iron kinetics in the RES using a physiologic RE tag was conducted by Fillet and co-workers (1989). Early RE radioiron release (from haem catabolism) was substantially increased in patients with haemochromatosis compared with subjects with similar iron stores. A defect in RE cells to retain iron freed from haemoglobin could result in an increased delivery of iron to the plasma. This would lead to high serum iron concentrations and increased loading of binding sites on transferrin. McClaren and co-workers (1991) in their study on intestinal iron absorption and mucosal iron kinetics in haemochromatosis, observed that the increased absorption can be attributed to an increased transfer rate (release) of mucosal iron in this disorder.

Since both mucosal cells and RE cells do not store iron appropriately, it was suggested that a common pathway of enhanced iron release may be operative in these tissues.

In conclusion, it appears that the only unifying hypothesis to account for the defect in HLA-linked haemochromatosis is that the RE cells are unable to hold iron leading to excess iron in the circulation, an increased transferrin saturation and an increase in non-transferrin-bound iron. How the release of iron is accomplished and in what form the iron is released is not known. Nevertheless, it suggests that this may be a disorder of iron release at a primary level and that the iron overload leading to toxicity is merely a secondary event. Furthermore, it appears that in order to clarify the basic lesion of haemochromatosis the abnormal gene or the abnormal gene product has to be identified.

1.6 AIMS OF THE PRESENT STUDY

The investigation of patients with HLA-linked haemochromatosis was of particular interest in this study because the reason why RE cells exhibit lesser degrees of iron overload compared to parenchymal cells, is not known. Macrophages are widely regarded as cells of the RES. Peripheral blood monocytes are precursors of macrophages and are easy to isolate and culture for experimental investigation. The attention of this study has therefore been focussed on certain aspects of cellular iron metabolism involving the *in vitro* culture of macrophages

for 8 days from patients with hereditary haemochromatosis.

A prerequisite for performing experiments on the cell population chosen, was to ensure that macrophages from normal and haemochromatotic subjects remained viable after 8 days in culture. To this end, a study was conducted to determine viability of these cells by trypan blue dye exclusion and to determine metabolic integrity (as a measure of viability) of uridine and leucine incorporation into RNA and protein, respectively.

It has been postulated that an impaired iron uptake by mononuclear phagocytes may be responsible for the uneven distribution of iron noted in HLA-linked haemochromatosis. The available information on transferrin iron uptake by monocytes and in particular cultured macrophages in this disorder is sparse. A study was aimed at comparing the handling of transferrin and transferrin iron by 8 day cultured macrophages from normal subjects and patients with hereditary haemochromatosis.

An abnormality in the regulation of ferritin synthesis has also been proposed as a possible mechanism accounting for the increased iron output by RE cells in hereditary haemochromatosis. To gain an insight into the expression of iron-regulated genes (ferritin H and L subunits) a study was aimed at directly measuring the level of early gene products (i.e., H- and L-ferritin mRNAs) in response to iron in 8 day cultured macrophages. Prior to the investigation of ferritin mRNA,

preliminary experimental methods were aimed at comparing various RNA isolation techniques from cultured macrophages. Although the central theme of this dissertation revolves around some aspects of cellular iron metabolism in haemochromatotic macrophages, the refinement and application of laboratory procedures were regarded as the cornerstone of this study.

SECTION 2

CELL BIOLOGY OF HAEMOCHROMATOTIC MACROPHAGES : GROWTH CHARACTERISTICS, VIABILITY STUDIES AND IRON METABOLISM.

SECTION 2

CHAPTER 2

BASIC CELL BIOLOGY METHODS

2.1 INTRODUCTION

Various experiments including cell viability, transferrin iron uptake and ferritin mRNA studies performed on cultured mature macrophages of patients with hereditary haemochromatosis and normal subjects are presented in this dissertation. Chapter 2 describes basic methods for the isolation of peripheral blood monocytes, their culture and growth characteristics. Viability studies as assessed by trypan blue exclusion, uridine and leucine uptake by cultured macrophages are also presented here. Chapter 3 deals with transferrin-iron-macrophage interactions. For convenience of presentation the determination of ferritin mRNA expresssion in cultured macrophages is discussed in section 3 (chapters 4 and 5) as this is mainly concerned with molecular biological techniques.

2.1.1 Subjects studied

The studies for this dissertation were approved by the University of the Witwatersrand Committee for Research on Human Subjects (protocol no. 15/8/89). Portions of blood samples taken during phlebotomy therapy of patients with haemochromatosis admitted to the Johannesburg Hospital, were used for the purpose of this study. Normal healthy volunteers from the Department of Medicine served as controls. Informed consent was obtained prior to the investigations. In total 8 haemochromatotic patients and 8 normal subjects were studied.

2.1.1.1 Diagnosis of haemochromatosis

The diagnosis of hereditary haemochromatosis was based on an elevated serum ferritin (> 250 μg/l), serum iron (> 150 μg/dl) and transferrin saturation (> 60%), on an increased hepatic non-haem iron index (> 2) and on compatible family studies (Ref. values: Powell et al, 1994; Bothwell et al, 1995). In addition, patients exhibited a wide variety of physical features characteristic of the disease. No patient had received blood transfusions or iron therapy and there was no evidence of excessive alcohol intake.

2.1.1.2 Laboratory assessment of subjects

Blood specimens drawn from each subject were used for the following investigations:

(i) Full blood count and differential count on an H1 electronic counter (Technicon, Terrytown, New York)

(ii) Serum iron concentration and total iron binding capacity (International Committee for Standardisation in Haematology, 1978 a&b)

(iii) Serum ferritin concentration measured by an enzyme linked immunoadsorbent assay (Conradie and Mbhele, 1980)

2.2 CELL CULTURE TECHNIQUES

2.2.1 Isolation of blood mononuclear cells

Mononuclear cells were isolated from whole blood by density gradient centrifugation on Ficoll-hypaque using a

modification of the technique described by Boyum (1968). In this technique, blood is spun on a fluid of high density (Ficoll-hypaque) resulting in the separation of blood cells into an upper fraction consisting of mononuclear cells and platelets at the interface and a sedimented fraction containing granulocytes and erythrocytes.

Blood removed by venesection from 8 normal volunteers and 8 patients with haemochromatosis was collected in 500 ml vaculiter containers with 70 ml citrate as anticoagulant. Serum samples were prepared by allowing freshly drawn blood to clot in 10 ml vac-u-test tubes. The citrated blood was mixed with an equal volume of sterile 0,9% saline as recommended by Boyum (1968). 33 ml of diluted blood was carefully layered onto 17 ml Ficoll-hypaque in 50 ml sterile screwcap tubes (Nunc). Layering of blood with minimal disturbance at the interface was achieved by tilting the tube from the horizontal and allowing filling to proceed slowly. The use of a pipette, as described by Boyum (1968), a syringe or a tap attached to a sterile tube proved to be time-consuming and laborious. The tubes were then centrifuged at 1400 rpm for 30 minutes at 4°C. The upper plasma layer was carefully removed and the mononuclear cell fraction was collected from the interface using a Pasteur pipette. The cells were washed 3 times by centrifugation at 1000 rpm for 20 minutes at 4°C using cold sterile RPMI medium (Highveld Biological, Johannesburg, South Africa) for removal of

platelets and Ficoll-hypaque. The resulting mononuclear cell pellet was resuspended in an appropriate volume of Basal Medium Eagle serum saver (BMESS) (Highveld Biological, Johannesburg, South Africa) with 10% autologous serum. After adequate mixing, 10 μl of the cell suspension was diluted in 90 μl white cell staining fluid (0.025% methyl green, 1% glacial acetic acid, in distilled water) and counted in a standard haemocytometer chamber. Cell counts were performed in duplicate.

2.2.2 Monocyte purification and macrophage culture

Numerous methods have been employed for the purification of monocytes from peripheral blood. Monocytes can be separated from lymphocytes by adherence to tissue culture dishes (Herscowitz et al. 1981). This technique has been widely applied owing to its simplicity and speed of performance. Other techniques for monocyte purification include density gradient centrifugation in Percoll (Feige et al. 1982) and centrifugal elutriation (Andreesen et al. 1990). In the present studies, the adherence method was used.

Aliquots of the cell suspension containing 3 x 10^6 mononuclear cells were added to 35mm diameter tissue culture grade petri dishes containing 2,5 ml BMESS supplemented with 10% autologous serum, 100 μg/ml streptomycin (Sigma) and 100 IU/ml penicillin (Sigma). When kept in culture for a length of time, these monocytes develop general features of macrophages (Zuckerman et al,

1979; Johnstone, 1988). It is well known that culture conditions may have a profound influence on the monocyte differentiation process and macrophage function *in vitro*. For example, monocytes cultured on fibronectin or collagen coated surfaces can modify the long term differentiation of monocytes (Kaplan and Gaudernack, 1982). Musson (1983) observed that monocytes cultured *in vitro* displayed changes dependent on the concentration of autologous serum and the length of time in culture, judged by several criteria. Various other functional, metabolic and morphological alterations associated with the terminal maturation of monocytes into macrophages *in vitro*, have been investigated. Increases in lysosomal enzymes (Yatziv *et al.* 1978; Musson *et al.* 1980), in cytotoxicity (Hammerstrom, 1979; Rinehart *et al.* 1979), in transferrin receptors (Andreesen *et al.* 1986; Hirata *et al.* 1986; Taetle *et al.* 1986) and in size and metabolism (Johnson *et al.* 1977; Zuckerman *et al.* 1979) have been reported. In view of the fact that macrophages are sensitive to the culture environment once removed from their natural location, it was necessary to optimize conditions for their cultivation. For the purpose of most of the experiments performed, macrophages were most conveniently maintained on 35mm diameter petri dishes at plating densities of 3×10^6 mononuclear cells per dish. The exclusion of serum resulted in detachment of the macrophages from the dishes after 2 to 3 days and a reduction in cell viability as assessed by trypan blue exclusion

[chapter 2.3.2]. Similar results were obtained upon the addition of fetal calf serum to the culture medium. Although a number of investigators have successfully cultured macrophages in vitro by using fetal calf serum, Johnson and co-workers (1977) reported that fetal or newborn calf, dog and horse sera did not support monocyte cultures for longer than 2 to 4 days. In the present studies, macrophages were maintained in 10% autologous serum. The cells were then incubated for a period of 8 days at 37°C in an atmosphere of 5% CO_2. It has been shown that monocytes kept in culture for 3 to 10 days develop the general features of tissue macrophages (Schlesinger et al, 1984). The number of cultured monocytes per dish could not be quantitated accurately. However, it was ensured that the plating density of mononuclear cells in each petri dish was the same in all subsequent experiments performed. Furthermore, to ensure successful cell culture all reagents were sterilized by filtering or autoclaving and all procedures were carried out with the use of a sterile hood.

2.2.3 Identification of cultured monocytes

2.2.3.1 Morphology and Photography

Monocytes obtained from haemochromatotic patients and normal subjects were cultured under identical conditions as described above [chapter 2.2.2]. Morphologic characteristics were assessed by phase contrast microscopy and photographed with an inverted Olympus PM 6 Camera (Tokyo)

attached to the phase contrast microscope (Olympus CK, Tokyo). After 12 to 24 hours in culture the monocytes assumed characteristic features of macrophages. The macrophages were well spread, round to oval with some cells developing cytoplasmic extensions. After 8 days in culture the macrophages were heterogenous in terms of morphology, nucleation and density and the mean cell diameter ranged between 30 to 40 μm (figures 1 and 2). For more accurate identification of monocytes, techniques demonstrating cytochemical staining characteristics and phagocytic abilities are preferred.

2.2.3.2 Esterase staining

The non-specific esterase staining method was performed as described by Yam and co-workers (1971). The monocytes stained reddish-brown and lymphocytes green. More than 90% of the adherent cells stained positively with non-specific esterase.

2.2.3.3 Phagocytosis

The phagocytic properties of monocytes were assessed by phagocytosis of Candida albicans (Miller, 1969). The yeast particles were inoculated in brain-heart infusion liquid medium and incubated at 37°C for 24 hours. The cell suspension was heat inactivated at 100°C for 30 minutes and then washed in phosphate buffered saline (PBS). The cells were counted in a haemocytometer chamber and 0,5 ml (10^9 yeast cells per ml) was incubated with 0,5 ml autologous serum at 37°C for 1 hour. The suspension was added in a ratio of 100 yeast cells per monocyte to the petri dishes

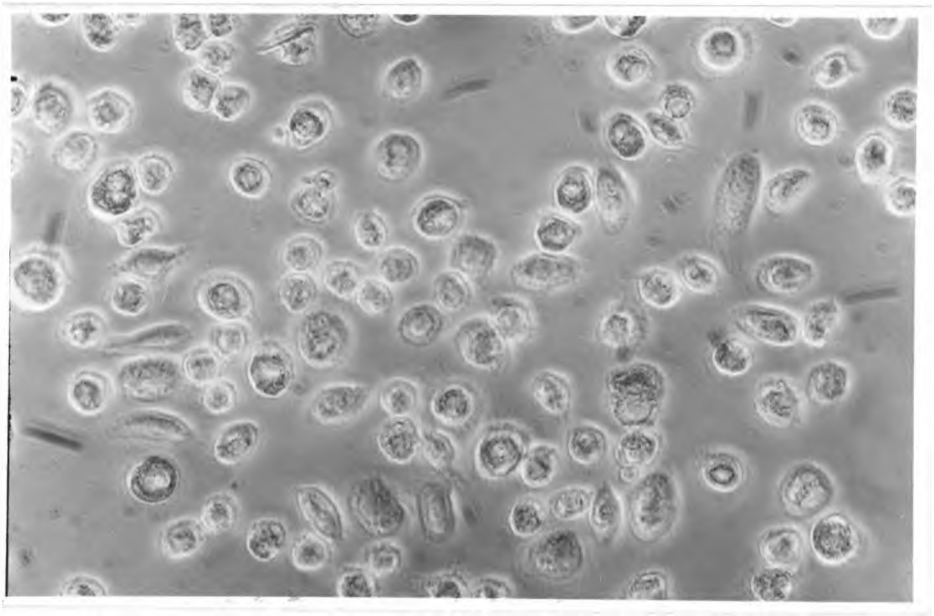

Figure 1: Phase contrast photography showing the morphologic characteristics of 8 day cultured macrophages derived from a normal subject.

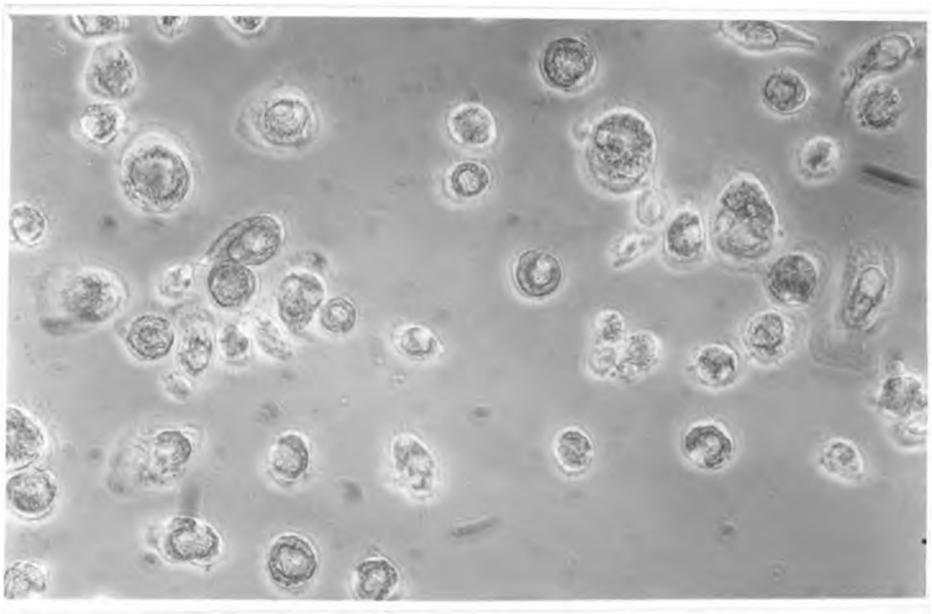

Figure 2: Phase contrast photography showing the morphologic characteristics of 8 day cultured macrophages derived from a patient with haemochromatosis.

containing the adherent population and incubated at 37°C for 1 hour. Thereafter the dishes were washed 3 times with PBS and the number of phagocytic cells counted. More than 80% of the adherent monocytes phagocytosed yeast particles.

2.3 ASSESSMENT OF CELL VIABILITY

2.3.1 Introduction

The viability of cells in culture can be assessed by cytological staining e.g. exclusion of trypan blue dye from viable cells. This method relies upon the integrity of cell membranes as a measure of cell survival. Other procedures based upon protein synthesis are probably better as these reflect directly on metabolic activity. The morphological and functional characteristics of a cell are largely determined by their macromolecular constituents such as proteins, carbohydrates and lipids, all of which are ultimately controlled by the nucleic acids. In in vitro tracer techniques, the addition of labelled nucleosides or amino acids to cells may be useful in analyzing the renewal of these macromolecular components. This study aimed to investigate the metabolic integrity of the adherent macrophage population after 8 days in culture. For this purpose the uptake of the tritiated (^{3}H) nucleoside, uridine, into RNA was determined concurrently with the incorporation of ^{3}H-leucine into protein of cultured macrophages derived from both haemochromatotic patients and healthy volunteers. The assessment of these

two functional parameters was considered important to clarify as a preliminary step to any subsequent studies to be undertaken on the cultured macrophages.

2.3.2 Subjects and Methods

For each study 2 haemochromatotic patients undergoing phlebotomy therapy and 2 normal volunteers were included. 300 ml citrated blood was separated by Ficoll-hypaque centrifugation to yield total mononuclear cells [chapter 2.2.1]. The resulting cell suspension was then added in aliquots of 3 x 10^6 cells per 35mm petri dish containing 2,5 ml BMESS with 10% autologous serum and antibiotics. The cells were incubated at 37°C under 5% CO_2 in air. All experiments were performed on "mature macrophages" after 8 days in culture. The cellular integrity of the initial mononuclear cell preparations and cultured adherent cells was firstly determined by exclusion of trypan blue (Mishell and Shiigi, 1980). The nonadherent cells and culture medium were removed by repeated washings with PBS and the adherent cells were gently removed using a rubber policeman. A solution of trypan blue in normal saline (1%) was added to a sample of cells and an aliquot was transferred to a standard haemocytometer chamber. Viable and non-viable (blue stained) cells were counted and the procedure was repeated to ensure accuracy. Greater than 98% of the adherent cells excluded trypan blue both at the outset and at the completion of the experimental time periods. More than 90% of the cells stained positively

with non-specific esterase and phagocytosed yeast particles. Culture dishes were set up in quadruplicate for each time interval. Control dishes containing no cells were also included. The culture medium and any nonadherent cells were removed from the petri dishes on day 8 by washing 3 times with PBS. Two ml of prewarmed RPMI medium containing 10% fetal calf serum was added to each dish containing adherent macrophages and to the dishes containing no cells and then incubated for 1 hour at 37°C. For the uridine uptake study, 2 μCi of [5,6 - ^{3}H]uridine (40 Ci/mmol, Amersham) was added to each dish. In a separate set of experiments, 20 μl of L-[4.5-^{3}H]leucine (143 Ci/mmol, Amersham) was added per dish so that the final concentration of radioactivity was 0,37 MBq/ml. The dishes were incubated at 37°C in a 5% CO_2 atmosphere. At timed intervals, the supernatant extracellular fluid was discarded and the adherent cells were washed 3 times with ice-cold PBS. One ml of a 0.2% Triton-100 and 0,1 M sodium hydroxide solution was added to each dish and after incubation for 5 minutes at room temperature, the lysed macrophages were transferred to test tubes. Each dish was washed twice more with 500 μl of the detergent solution. To each tube, containing 2 ml of resultant lysate and washes, was added either 100 μl of unlabelled uridine (1 mg/ml) or 100 μl of bovine serum albumin (1%) for the leucine uptake study. The lysate was made up to 5% trichloroacetic acid and the tubes were vortexed briefly and chilled on ice. The precipitates were collected on

glass microfibre discs (Whatman) using a Millipore filter apparatus. Each tube was washed twice more with ice-cold 5% trichloroacetic acid. The filter discs were immersed in 5 ml scintillation cocktail and the trichloroacetic acid-precipitable radioactivity was measured in a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3375, Illinois, USA). Corrections were made for background counts by counting unused discs. Results were expressed as mean counts per minute (cpm) ± standard error (SE) of ^{3}H-uridine and ^{3}H-leucine incorporated into RNA and protein respectively, per dish.

2.3.3 Results

The experimental findings for the uridine uptake study are depicted in figure 3. The incubation of 8 day-old cultured macrophages with ^{3}H-uridine led to a steady accumulation of the pyrimidine base over a time period of 8 hours. It can be seen that the extent of uridine incorporation by haemochromatotic macrophages was comparable to that noted in normal macrophages.

The results of representative experiments for the leucine uptake study (figure 4) demonstrate a linear uptake of the amino acid by the cultured macrophages with respect to time. At 3 hours, a three-fold increase in the ^{3}H-leucine was detected in cells from both normal individuals and patients with haemochromatosis. The results reported here show that in general, the time

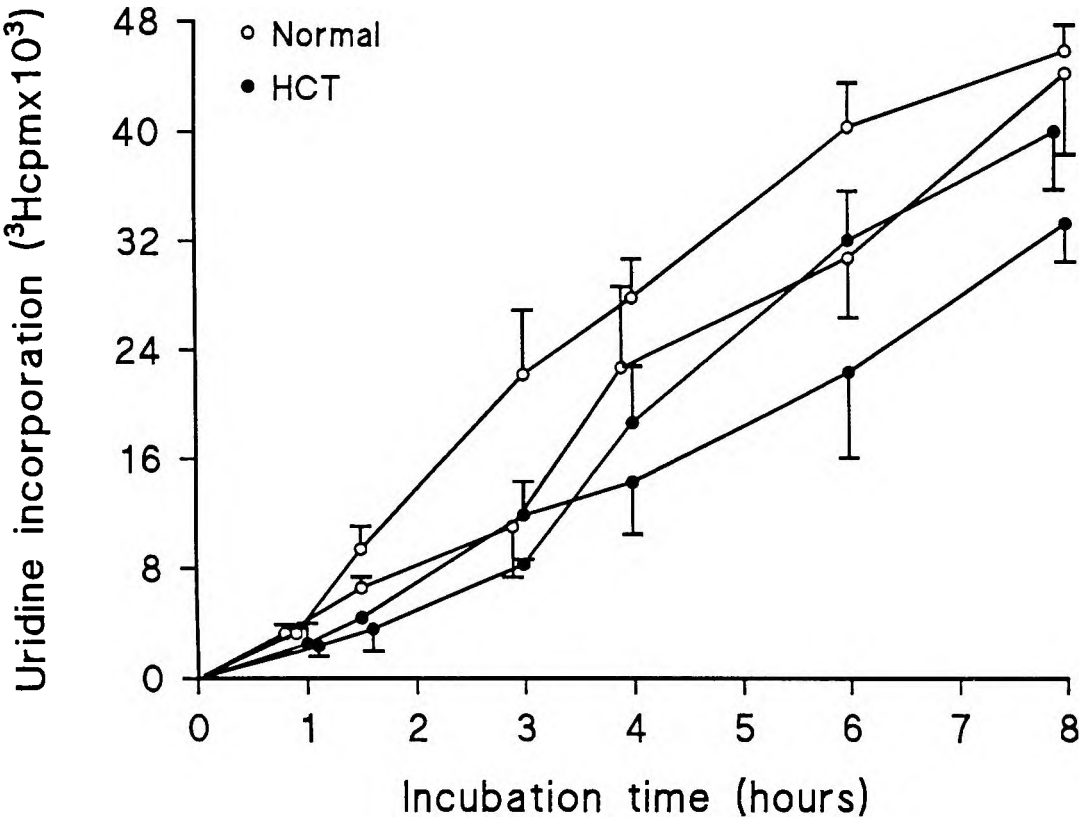

Figure 3: The time-dependent uptake of ^{3}H-uridine by 8 day cultured macrophages derived from 2 haemochromatotic (HCT) patients (●) and 2 healthy subjects (o). Each point represents the mean ± SE of quadruplicate culture dishes.

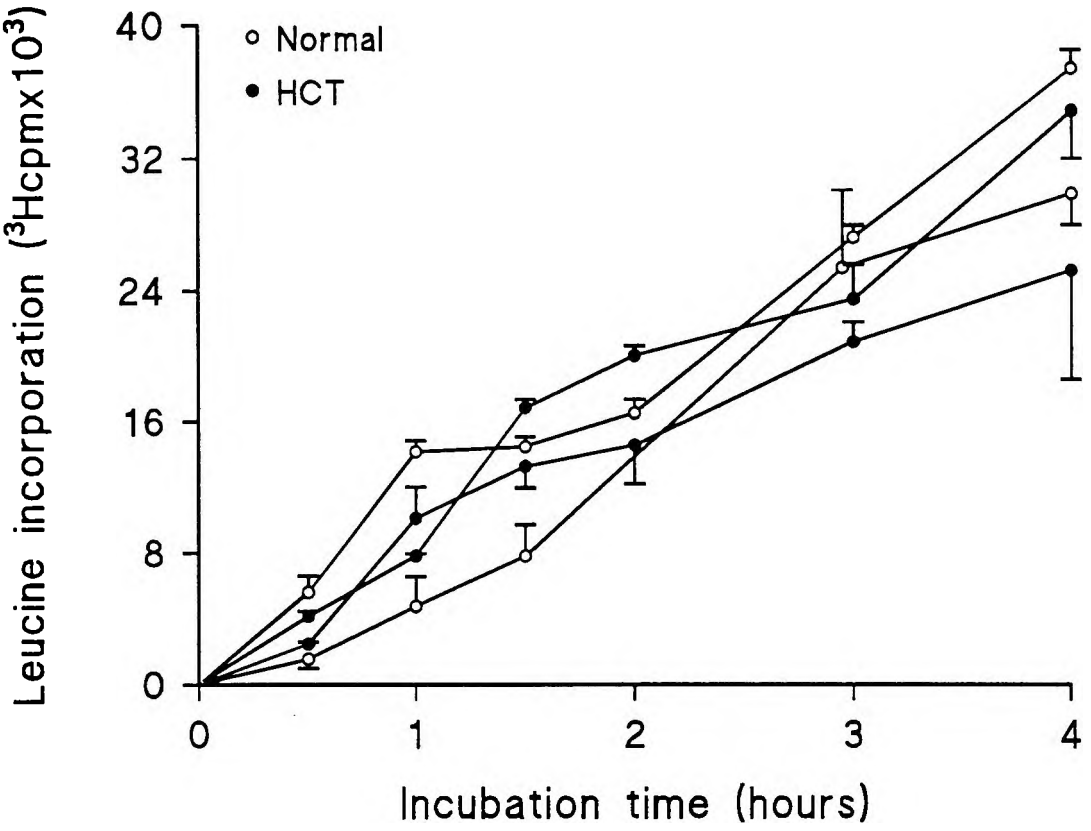

Figure 4: Time course of the incorporation of ^{3}H-leucine in haemochromatotic (●) and normal (o) macrophages after 8 days in culture. Each point represents the mean ± SE of quadruplicate dishes.

course for the incorporation of ^{3}H-leucine into newly synthesized protein was very similar for both study groups investigated.

It was decided not to pursue these studies further based on the essential similarities observed in the graphs of both groups.

2.3.4 Discussion

The investigation of the incorporation of precursor molecules into RNA and protein was initiated to assess whether macrophages maintained in culture for 8 days still attained metabolic activity. As discussed before [chapter 2.2.2] numerous studies on human monocytes have revealed biochemical, morphologic and functional alterations during their *in vitro* maturation. In the current investigations, the addition of a precursor amino acid such as leucine to the incubating medium of macrophages relatively deprived of nutritious agents after 8 days in culture, resulted in a progressive increase in the total protein synthesis of the adherent population. Similarly the RNA synthetic rates progressed linearly with respect to time upon addition of uridine. It was also observed that the uptake of uridine and leucine for nucleic acid synthesis and protein synthesis respectively, was similar for the study groups investigated.

In conclusion, the present results indicate that cultured macrophages accept uridine and leucine from the extracellular environment and retain their ability to

synthesize RNA and protein after 8 days in culture, showing that the cells are still metabolically viable. Furthermore, these data show that whatever changes occur in monocytes upon adherence, the rates of protein synthesis and RNA synthesis are not different between normal and haemochromatotic cells by day 8.

SECTION 2

CHAPTER 3

TRANSFERRIN-IRON-MACROPHAGE INTERACTIONS

3.1 INTRODUCTION

The relative paucity of iron deposition in cells of the RES in HLA-linked haemochromatosis as compared with parenchymal cells of affected tissues (Valberg et al, 1975; Brink et al, 1976; McClaren, 1989), has long been noted. Possible defects in RE iron uptake and storage have been extensively investigated, but to date not been fully clarified.

Monocyte-macrophages and other cells of the RE system play a central role in the storage of iron, which is mostly acquired from the phagocytosis of senescent red cells (Bothwell et al, 1979). The presence of receptors for diferric transferrin on human macrophages (Andreesen et al, 1983; Adams and Hamilton, 1984; Andreesen et al, 1984; Baynes et al, 1987b) suggests an alternative source of iron for RE cells. The relative absence of RE iron in haemochromatosis has stimulated interest in a possible abnormality in the uptake of transferrin iron by RE cells. Such a defect could result in a high level of iron absorption and plasma transferrin saturation with the deposition of excess iron in parenchymal tissues (Cook et al, 1973). Several studies have been unable to support this hypothesis. In vitro studies on peripheral blood monocytes have shown no difference in transferrin iron uptake between phlebotomised patients with normal body iron stores and normal control subjects (Jacobs and Summers, 1981; Sizemore and Bassett, 1984). However, a significant reduction in transferrin iron uptake by

monocytes has been reported in both treated (McClaren et al, 1979) and iron-loaded haemochromatosis patients (Sizemore and Bassett, 1984). The differences observed in transferrin iron uptake in haemochromatosis by various authors has led to investigations into abnormalities in transferrin receptor binding and transferrin receptors. Studies have failed to reveal any differences in the properties of transferrin receptors on cultured fibroblasts and lymphocytes (Ward et al, 1984a) or macrophages (Baynes et al, 1989) derived from haemochromatotic patients. Contradictory evidence demonstrating increased transferrin receptor expression on monocytes from patients with hereditary haemochromatosis has been provided by Bjorn-Rasmussen and colleagues (1985). It is clear that the available data on transferrin-iron-macrophage interactions in HLA-linked haemochromatosis is not consistent.

In the present in vitro study, radiolabelled diferric transferrin uptake was examined in cultured macrophages in order to determine whether the behaviour of haemochromatotic macrophages differed from normal macrophages.

3.2 SUBJECTS AND METHODS

The study group comprised 3 haemochromatotic patients undergoing phlebotomy therapy and 3 healthy volunteers. The serum iron concentration, total iron-binding capacity and serum ferritin concentration were assessed in all subjects according to methods described in chapter

2.1.1.2. Student's t tests were applied to compare the means of the biochemical measurements. Doubly labelled diferric transferrin was prepared according to established methods. Human transferrin was obtained from Sigma (St Louis, Missouri, USA), ^{59}Fe as $FeCl_3$ and ^{125}I as Bolton and Hunter reagent were from Amersham (Buckinghamshire, UK). Diferric transferrin was prepared by labelling transferrin with ^{59}Fe as described by Baynes and co-workers (1987b). The degree of iron saturation was evaluated by spectroscopy and on 6 M urea polyacrylamide gel electrophoresis using the method of Makey and Seal (1976). The ^{59}Fe labelled differic transferrin was then iodinated with ^{125}I by the Bolton and Hunter Method (1973). The end concentration of diferric transferrin was determined by radial immunodiffusion. To remove any contaminating iron, all buffers and media were prepared with distilled water that had been extracted with 200-400 mesh Chelex 100 resin (Biorad, Richmond, California, USA).

Peripheral blood mononuclears were isolated from 300 ml citrated blood and cultured as previously described [chapter 2.2]. Cultures were established at 37°C in a 5% CO_2 incubator by accurately plating 3 x 10^6 cells onto 35mm diameter tissue culture dishes containing 2,5 ml BMESS with 10% autologous serum and antibiotics. Both dose and time response studies were performed on the eighth day of culture. At this time, the viability of the adherent cells was more than 98% as judged by trypan blue exclusion, while more than 90% stained positively with non-specific

esterase and phagocytosed yeast particles. The culture medium and any nonadherent cells were removed from the petri dishes and the dishes were washed 3 times with PBS. Thereafter, 2 ml RPMI medium and 1% bovine serum albumin were added and the dishes incubated for 1 hour at 37°C. After the preincubation period, doubly labelled diferric transferrin was added to each dish in specific amounts (see figure legends) for the dose response studies, while a constant amount was added in the time response experiments. Control dishes containing no cells were included in each study. Petri dishes containing a 100-fold excess of unlabelled diferric transferrin were also set up for each dose and time component. The dose-dependent diferric transferrin studies were performed over 90 minutes, whereas various time intervals were chosen for the time-dependent studies. On completion of the incubation at 37°C / 5% CO_2 atmosphere, the supernatants were removed and the adherent cells were washed 3 times with ice-cold PBS. One ml of a 0,2% Triton-100 and 0,1 M sodium hydroxide solution was added to each dish and incubated for 5 minutes at room temperature. The cellular lysates were collected in test tubes. Two additional washes of the petri dishes with the detergent solution were also collected. The resultant suspension of disrupted cells was counted for ^{125}I and ^{59}Fe activity in a Packard Autogamma counter (Model 5650, Packard Instruments, Downers Grove Illinois, USA) in order to determine diferric transferrin and iron uptake respectively. Appropriate corrections were

made for cross counting and any non-specific interactions between the transferrin and the petri dishes. The total experimental binding less the counts obtained from dishes without cells and less the counts obtained from dishes which also had a 100-fold excess of unlabelled diferric transferrin was calculated in order to obtain cell specific binding. The results are expressed as pmoles transferrin or iron uptake per mg of protein. The results for the time response studies are given as means and standard deviations for the experiments performed on 3 patients with haemochromatosis and 3 normal subjects. (Within an experiment determinations were carried out in triplicate). Group means at each time point were compared by using Student's t test. The protein content per dish was assayed according to the dye binding method (Biorad, Richmond, California, USA) using bovine serum albumin as standard. Here the mean figure of quadruplicate dishes containing cells derived from each subject was determined.

3.3 RESULTS

The biochemical features of the study participants are summarized in Table I. The mean serum ferritin (SF), serum iron (SI) and transferrin saturation concentrations of haemochromatotic patients were within or slightly below the normal range as a result of a vigorous phlebotomy programme. There were no significant differences between normal and haemochromatotic groups when Student's t tests were applied to all biochemical measurements.

TABLE I: Mean and SD for serum iron (SI), serum ferritin (SF), transferrin iron binding capacity (TIBC) and % transferrin saturation for 3 normal subjects and 3 patients with haemochromatosis undergoing phlebotomy therapy. The level of significance of difference between measurements as determined by Student's t test is shown.

STUDY GROUP	SI(μg/dL)	SF(μg/l)	TIBC(μg/dL)	%TFSAT
Normal subjects				
1	82	35	271	30.3
2	82	99	280	29.3
3	153	52	327	46.8
Mean	105.7	62	292.7	35.5
± SD	41	33.2	30.1	9.8
HCT patients (treated)				
1	49	11	195	25.1
2	104	16	316	32.9
3	131	21	460	28.5
Mean	94.7	16	323.7	28.8
± SD	41.8	5	132.7	3.9
t-value *	0.33	2.38	0.395	1.94
p value *	0.76	0.14	0.71	0.12
Normal range	60-150	20-250	250-400	20-50

* Normal and haemochromatotic (HCT) groups statistically equivalent by Student's t test for all parameters.

The results of the dose-dependent uptake of diferric transferrin by macrophages in patients with hereditary haemochromatosis and normal control subjects are shown in figure 5. Concentrations ranging between 5 to 250 pmoles of labelled diferric transferrin were added to each petri dish. During the 90 minute incubation period the specific uptake of the protein proceeded in a linear fashion up to approximately 60 pmoles of added transferrin and thereafter a plateau was reached. This was observed in macrophages from both study groups. The binding characteristics were not assessed in this study.

Based on the results of the dose response study, a constant amount of 50 pmoles of labelled differic transferrin was added to each dish in the time-dependent uptake studies that followed. The measurements of transferrin uptake by macrophages from both study groups and their comparative statistical values are listed in Table II. Figure 6 shows the uptake of ^{125}I-diferric transferrin by macrophages in the 3 healthy subjects and 3 patients with haemochromatosis. The time course study (figure 6) revealed that there was a progressive uptake of transferrin at first and then maximal uptake was achieved by 90 to 180 minutes. The mean transferrin uptake by haemochromatotic macrophages was about 1.9 times greater at 1 hour ($p = 0.003$) and 1.4 times greater at 1.5 hours ($p = 0.009$) than normal macrophages (Table II). There was no significant difference between normal subjects and haemochromatotic patients with respect to mean transferrin uptake at 4 hours ($p = 0.053$).

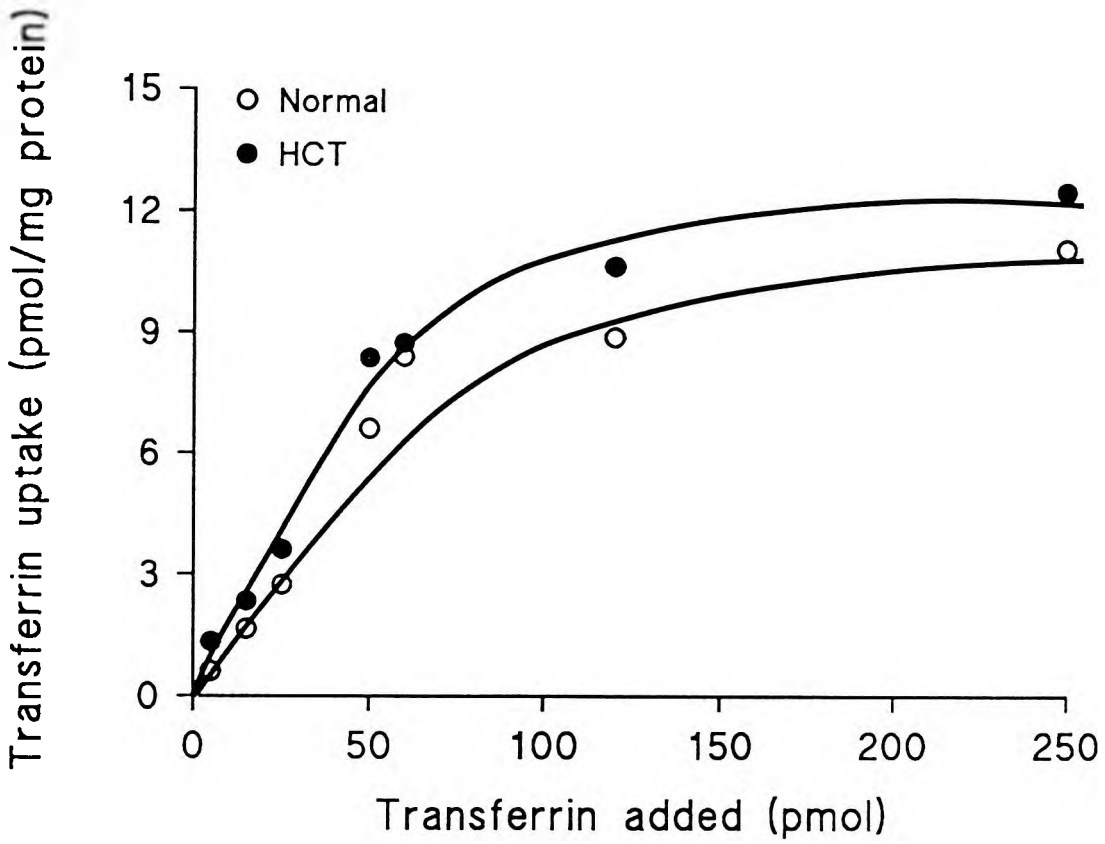

Figure 5: The dose-dependent uptake of diferric transferrin by haemochromatotic (●) and normal (o) cultured macrophages. The duration of incubation in the presence of labelled diferric transferrin was 90 minutes. Each dose point represents the mean value of triplicate culture dishes.

TABLE II: Mean and SD for transferrin uptake (pmole/mg protein) at various time intervals by cultured macrophages from 3 normal subjects and 3 treated haemochromatotic (HCT) patients. Group means at each time point were compared by Student's t test.

TIME (hr)	NORMAL (n=3)	HCT (n=3)	t-value	p value
0.5	1.45 ± 0.5	3.08 ± 0.43	4.3	0.013
1.0	2.54 ± 0.26	4.95 ± 0.43	8.4	0.003
1.5	3.63 ± 0.27	5.24 ± 0.34	6.0	0.009
2.0	4.13 ± 0.31	5.51 ± 0.25	5.2	0.04
3.0	4.45 ± 0.43	5.97 ± 0.41	3.9	0.03
4.0	4.41 ± 0.59	5.81 ± 0.66	2.7	0.053*

* Difference not statistically significant.
Differences were regarded as significant when $p < 0.05$. Although visually a difference is observed, the small number of subjects studied make the t-values unreliable.

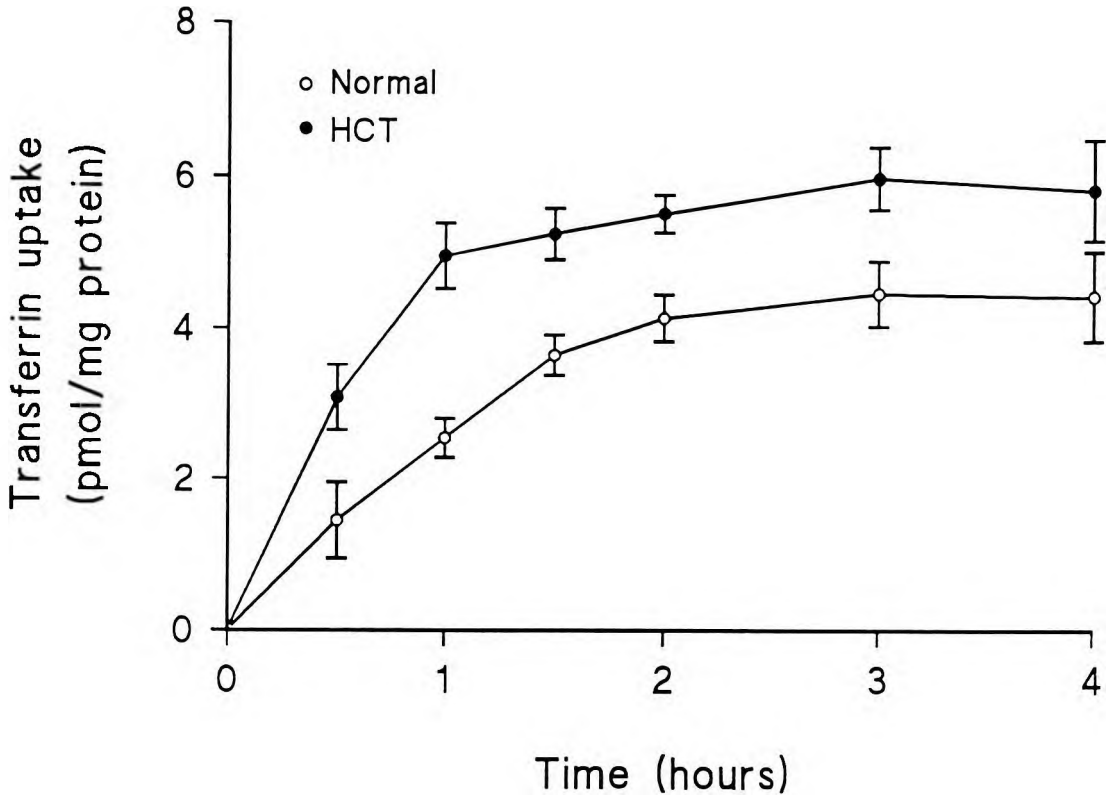

Figure 6: The time-dependent uptake of transferrin per mg of protein by cultured macrophages obtained from 3 haemochomatotic patients (●) and 3 normal subjects (o). Doubly labelled diferric transferrin (50 pmol) was added to each petri dish. Each time point represents the mean and standard deviation from 3 experiments.

In the studies evaluating the uptake of ^{59}Fe-diferric transferrin by macrophages, a linear increase in iron uptake was noted (figure 7). This pattern was observed in all study participants. The measurements for iron uptake by macrophages from both study groups and their comparative statistical values are shown in Table III. There was no difference in the mean cellular iron uptake at 30 minutes between normal subjects and haemochromatotic patients ($p = 0.34$), however iron uptake by haemochromatotic cells was about 2.7 times lower at 2 hours ($p = 0.01$) and 2.1 times lower at 5 hours ($p = 0.006$) than normal cells (Table III).

3.4 DISCUSSION

The current *in vitro* study was aimed at comparing transferrin and transferrin iron handling by peripheral blood monocytes derived from 3 treated haemochromatotic and 3 healthy subjects. The objective of treatment in patients with haemochromatosis is to reduce body iron stores by repeated phlebotomy. After venesection, storage iron is mobilized to satisfy erythroid marrow requirements. The results shown in Table I indicate that, at the time of the study, excess iron had been removed from the patients with haemochromatosis and the concentration of serum iron did not differ significantly from the control group. The serum ferritin concentration in these patients was bordering at the level generally accepted as indicating depleted iron stores (Bothwell *et al.* 1979),

TABLE III: Mean and SD for iron uptake (pmole/mg protein) at various time intervals from 3 normal subjects and 3 treated haemochromatotic (HCT) patients. Group means at each time interval were compared by Student's t test.

TIME (hr)	NORMAL (n=3)	HCT (n=3)	t-value	p value
0.5	21.16 ± 11.93	12.51 ± 6.37	-1.1	0.34*
1.0	45.36 ± 6.22	20.62 ± 11.08	-3.4	0.04
1.5	74.23 ± 11.61	27.20 ± 16.51	-4.0	0.02
2.0	94.97 ± 11.73	35.61 ± 19.74	-4.5	0.01
3.0	113.63 ± 14.16	56.73 ± 15.36	-4.7	0.009
5.0	150.38 ± 13.87	71.49 ± 19.62	-5.7	0.006

* Difference not statistically significant.

Differences were regarded as significant when $p < 0.05$. Although visually a difference is observed, the small number of subjects studied make the t-values unreliable.

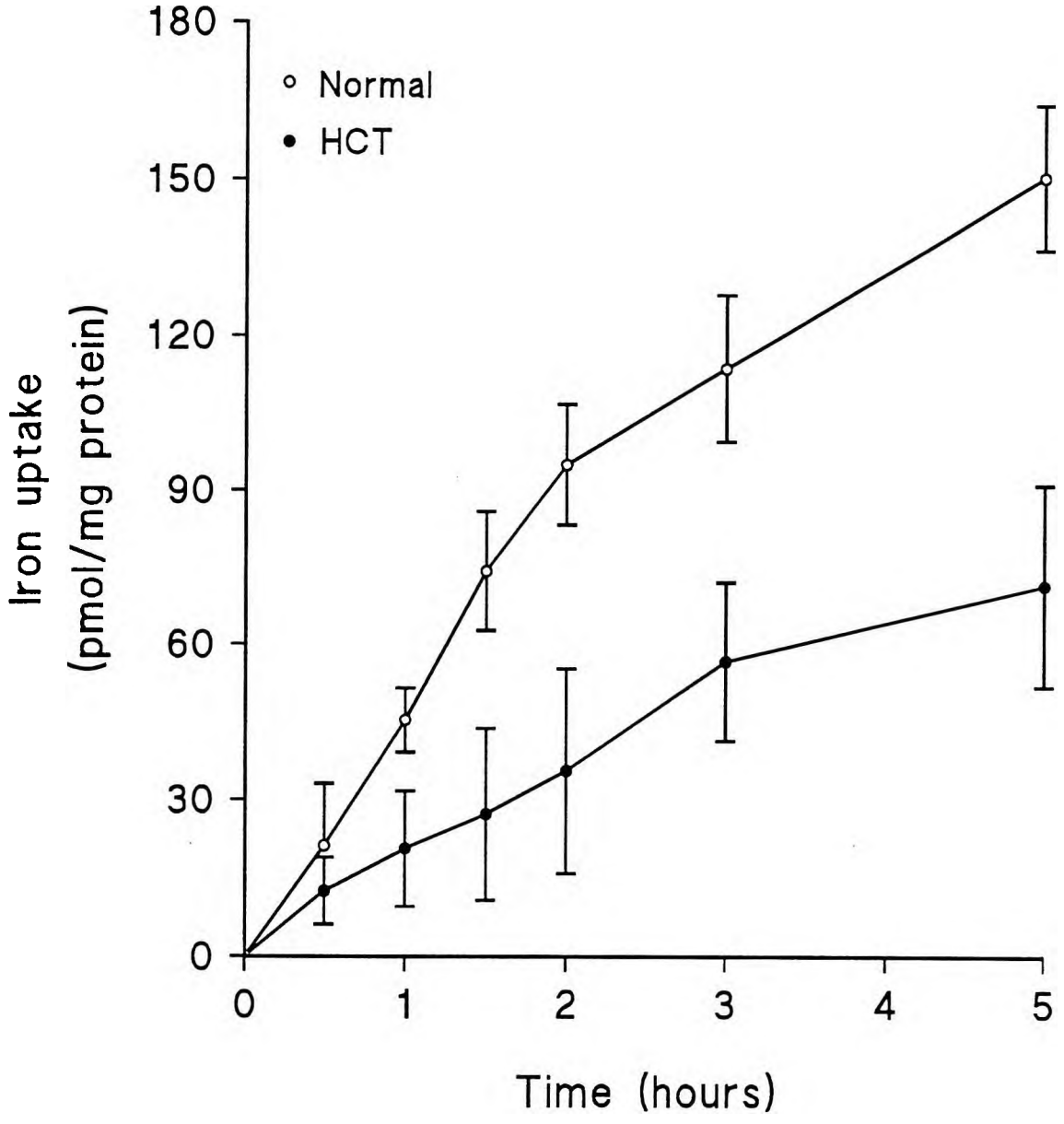

Figure 7: The uptake of iron by haemochromatotic (●) and normal (o) cultured macrophages as a function of time. Doubly labelled diferric transferrin (50 pmol) was added to each culture dish. Each time point represents the mean and standard deviation from 3 experiments.

but was not significantly lower than in the control group ($t = 2.38$, $p = 0.14$).

The results obtained in the in vitro experiments on transferrin-iron-macrophage interactions indicate that both normal and haemochromatotic macrophages possess a mechanism for the uptake of diferric transferrin. Uptake by haemochromatotic macrophages was 1.9 times greater (at 1 hour) and 1.4 times greater (at 1.5 hours) than uptake by normal macrophages (Table II & figure 6). While in this study binding characteristics were not evaluated, previous work has provided evidence for the presence of diferric transferrin receptors on macrophages (Andreesen et al. 1983 & 1984; Adams and Hamilton, 1984; Baynes et al. 1987b; Testa et al. 1989). Studies have shown no significant difference between the binding affinities of transferrin receptors on macrophages from haemochromatotic patients and normal subjects (Ward et al. 1984a; Baynes et al. 1989). The greater increase in uptake of diferric transferrin by the haemochromatotic macrophages observed in the present study may have been a secondary response to a low intracellular iron pool and an increased transferrin receptor expression through the IRP mechanism (section 1, chapters 1.2.5.3 and 1.3.6.2). Bjorn-Rasmussen and colleagues (1985) demonstrated an increased transferrin receptor expression on circulating monocytes from 12 haemochromatotic patients, but there was no correlation between the degree of patient iron overload and the number of transferrin receptors on monocytes. Although no

significant difference was found between normal subjects and haemochromatotic patients in their biochemical values (Table 1), it is possible that the observed difference in their uptake of diferric transferrin represents a real difference since studies (Testa et al, 1989; Byrd and Horwitz, 1993) have shown that transferrin receptor expression is upregulated by iron.

In the final part of the current study, the uptake of iron derived from transferrin was compared in normal and haemochromatotic macrophages. The uptake of transferrin iron was 2.7 times lower (at 2 hours) and 2.1 times lower (at 5 hours) when compared to normal macrophages (Table III & figure 7) despite the higher transferrin uptake by macrophages from patients with haemochromatosis (figure 6). These findings may be of physiological importance since the RE system in hereditary haemochromatosis does not normally retain iron (Valberg et al, 1975; Brink et al, 1976). The observed differences in transferrin iron uptake may be the result of a possible abnormality in post-receptor events. In this regard, abnormalities of either iron release or iron storage could account for this difference. There is some evidence of defective iron release in haemochromatosis. The investigations by Fillet and colleagues (1989) into iron kinetics in the RE system of humans using heat-damaged erythrocytes labelled with radioactive iron, identified an early iron release phase, from haem catabolism, and a late release phase, from RE iron stores. Early radioiron release was increased in

patients with haemochromatosis compared with subjects with similar iron stores. This was true both for patients with limited and markedly increased iron stores. A similar mechanism could explain why iron uptake by macrophages from haemochromatotic patients appeared to be less than controls in the present study, as iron, entering on transferrin, is more rapidly released than in normal macrophages. The relative paucity of stored iron in the RE system of haemochromatotic individuals, has also led many investigators to believe that synthesis of ferritin may be defective in macrophages. Many studies do not support this hypothesis. No defect in the ferritin synthesis of these cells has been found in haemochromatosis patients (Jacobs and Summers, 1981; Bassett et al. 1982). However, a recent report by Gordeuk and co-workers (1992) demonstrated an impaired release of tumor necrosis factor-α (TNF-α) from cultured blood monocytes from homozygous haemochromatotic patients. Of relevance to the present study is the authors suggestion that a reduced amount of TNF-α may result in inhibition of ferritin synthesis and enhanced release of iron from monocytes.

In summary, the current study constituted an attempt to identify any differences in transferrin and transferrin iron uptake by cultured macrophages between treated haemochromatotic patients and normal subjects. Cells from both groups were shown to take up diferric transferrin. Transferrin uptake was significantly higher in macrophages from patients with haemochromatosis. This was thought to

be due to a secondary effect to low intracellular iron levels or possibly due to increased transferrin receptor expression. The iron uptake was shown to proceed at a lower level in the cells from patients with haemochromatosis. An altered ferritin synthesis, ferritin content or enhanced release of iron by various mechanisms may be amongst several possible explanations for this effect. It must be emphasized that the results reported here represent a small number of subjects and more studies are needed on a larger group in order to verify these findings. Furthermore, while the results are suggestive of a deranged iron homeostasis in haemochromatotic RE cells in vitro, it should be noted that the uptake of transferrin iron by mononuclear cells represents a relatively insignificant pathway in the in vivo situation. Therefore, its relationship to the pathogenesis of HLA-linked haemochromatosis remains questionable.

SECTION 3

FERRITIN mRNA EXPRESSION IN HAEMOCHROMATOTIC MACROPHAGES

SECTION 3

CHAPTER 4

MATERIALS AND METHODS

4.1 INTRODUCTION

The present chapter is concerned with various molecular biological techniques. Three methods are presented for the extraction of RNA from human cultured macrophages. The preparation of plasmid DNA, restriction digestion, radiolabelling of DNA and hybridization are also described here. Many of the techniques, shown to have worked in this laboratory, have been modified or expanded to suit this particular study. The methodologies explored by Dr.S.E. Lapinsky in his study on reticuloendothelial messenger RNA in inflammatory states (1989) provided a valuable stepping stone for the practical aspects concerned in this chapter.

4.2 PREPARATION OF RIBONUCLEIC ACID

4.2.1 RNA isolation methods

In this section techniques are presented for the isolation of RNA from cultured macrophages. The extraction of undegraded ribonucleic acid requires that ribonuclease (RNase) contamination be minimized. To ensure RNase removal, all glassware was baked at 250°C for 4 hours; disposable pipette tips were autoclaved at 120°C for 30 minutes; whenever possible, stock solutions were sterilized by treatment with 0,1% (v/v) diethylpyrocarbonate (DEPC), an effective inhibitor of RNase, and autoclaved for 30 minutes at 120°C and gloves were worn during handling of all items and solutions in contact with the RNA. The stability of RNA was further ensured by keeping

all solutions ice-cold. Three methods were assessed for the isolation of RNA from macrophages cultured in vitro. The isolation of blood mononuclear cells and the culture of monocytes was followed as described in section 2, chapter 2.2. All RNA extraction procedures were performed on the eighth day of culture.

The guanidinium isothiocyanate method was first investigated for the isolation of RNA. Guanidinium salts are potent chaotropic denaturants of proteins, including ribonucleases. Cox (1968) described the use of guanidinium chloride for the isolation of RNA from ribosomes in which ribonucleases were denatured. For tissues with high RNase activity such as the pancreas, Chirgwin and co-workers (1979) made use of guanidinium thiocyanate as an effective deproteinizing agent. In the present study, the isolation of total RNA from macrophages was performed after modifications of the method of Chomczynski and Sacchi (1987). After removal of the medium and any nonadherent cells from the 35mm diameter tissue culture dishes, the dishes were washed 3 times with PBS. 200 µl of the denaturant solution (guanidinium isothiocyanate 4 M; sodium citrate 25 mM; sodium lauryl sarcosine 0,5% (w/v); ß-mercaptoethanol 0,1 M) was added to each dish. The surface was scraped with a rubber policeman and the dishes were washed with an additional 200 µl of denaturant solution. One ml aliquots were transferred to 2,2 ml Eppendorf tubes. To each 1 ml of solution was added 100 µl of sodium acetate (2 M, pH 4), 1 ml of DEPC-saturated phenol containing 8-hydroxy-

quinoline (0,1%) and 200 µl of a chloroform : isoamyl-alcohol solution (49:1). The contents of each tube were thoroughly mixed by vortexing for 30 seconds and then kept on ice for 15 minutes. The samples were centrifuged at 10 000 g for 20 minutes at 4°C. The upper aqueous phase, which contained the RNA, was carefully collected in 2,2 ml Eppendorf tubes and the phenol phase containing proteins and DNA was discarded. The RNA in the final aqueous phase was precipitated by adding an equal volume of isopropanol and chilled at -70°C overnight. The RNA was sedimented at 10 000 g for 20 minutes at 4°C and the pellet was resuspended in 75% ethanol. After centrifugation, the pellet was vacuum dried for 15 minutes and dissolved in a suitable volume of DEPC-treated water (15 to 30 µl).

The lithium chloride method for RNA isolation was next evaluated. The procedure, as described by LeMeur and colleagues (1981), was adapted for the extraction of RNA from cultured monocytes. Two ml of homogenisation buffer (lithium chloride 3 M; urea 6 M; sodium acetate 10 mM; heparin 200 µg/ml; sodium dodecyl sulfate 0,1% (w/v); ß-mercaptoethanol 1%) was added directly to each petri dish containing adherent macrophages. The cells were scraped off the dishes with a rubber policeman and the pooled contents of 3 dishes were homogenized using an Ultra-Turrax homogenizer. The resultant solution was aliquotted as 2 ml volumes into 2,2 ml Eppendorf tubes and left overnight at 4°C. The samples were then centrifuged at 10 000 g for 20 minutes at 4°C. The supernatant was carefully

removed and the pellet was washed 3 times by centrifugation with 2 ml of washing buffer (lithium chloride 4 M; urea 8 M; ß-mercaptoethanol 1%). The aqueous solution was removed and the pellet dissolved in 400 µl of solubilization buffer (sodium acetate 50 mM; sodium dodecyl sulfate 1% ; ß-mercaptoethanol 1%). Each sample was extracted twice by the addition of 400 µl phenol (Tris-EDTA saturated, pH 8,0) and 400 µl chloroform : isoamyl-alcohol (24:1). After thorough mixing by vortexing for 30 seconds, the samples were centrifuged at 10 000 g for 20 minutes at 4°C to separate the phases. The upper aqueous was carefully removed and to this an equal volume of isopropanol was added. After mixing, the samples were incubated at -70°C overnight. The RNA was pelleted once more by centrifugation, washed in 75% ethanol, vacuum dried and dissolved in DEPC-water.

The NP40-urea-SDS method was lastly investigated. This technique, suitable for the preparation of cytoplasmic RNA from tissue culture cells, was described by Gough (1988). The macrophages with their growth medium were harvested directly from the petri dishes by gently scraping the surface with a rubber policeman. The cells were pelleted in 1,5 ml Eppendorf tubes by centrifugation at 800 g for 5 minutes. The medium was removed by aspiration and 200 µl of an ice-cold Tris-HCL 10 mM (pH 7,5); magnesium chloride 1,5 mM; Nonidet P-40 0,65% (v/v) solution was added to each pellet. After vortexing vigorously, the preparation was centrifuged at 800g for 5

minutes at 4°C to remove nuclei. To 200 µl aliquots of the aqueous supernatant was added 200 µl of a solution containing urea 7 M; sodium dodecyl sulfate 1% ; sodium chloride 0,35 M; Tris-HCL 10mM (pH 7,5) and EDTA 10 mM. After mixing, 400 µl phenol : chloroform : isoamylalcohol (50:50:1) was added to each Eppendorf tube and then vortexed for 30 seconds. The samples were incubated on ice for 15 minutes and then centrifuged for 10 minutes at 10000 g. The RNA was recovered from the aqueous phase by precipitation with 2,5 volumes of 95% ethanol and chilled at -70°C overnight. The samples were centrifuged to sediment the RNA and the ethanol was evaporated by vacuum drying. The pellet was then dissolved in a small volume of DEPC-water. All RNA samples were stored at -20°C for future use.

Both the guanidinium thiocyanate and lithium chloride methods proved effective enough to provide good yields of RNA and the extracted RNA was pure and undegraded (see chapter 4.2.3, figures 9 and 10). It is of paramount importance that all steps in the isolation of RNA are performed as quickly as possible. In this regard, the lithium chloride method is time-consuming whereas the use of guanidinium thiocyanate is a relatively simple procedure permitting the rapid recovery of RNA from cultured cells. The NP40-urea-SDS method followed according to Gough (1988) was unsuccessful as evidenced by the electrophoretic pattern demonstrating the presence of degraded RNA [chapter 4.2.3, figure 11]. It is possible

that the use of sodium dodecyl sulfate and urea were insufficiently effective as protein denaturants, although Gough (1988) was able to extract intact RNA from tissue culture cells and cell suspensions. The method of choice for the isolation of RNA from cultured macrophages for all future in vitro experiments, was the guanidinium thiocyanate-phenol-chloroform extraction.

4.2.2 Spectroscopic analysis

The RNA was quantitated spectrophotometrically by measuring the amount of ultraviolet (uv) irradiation absorbed at 260 nm and using the extinction coefficient of A = 1.0 for 40 μg RNA/ml [1 cm light path] (Maniatis et al, 1982). The absorbance was also recorded at 280 nm. The ratio of A_{260nm} / A_{280nm} is a useful indicator of the purity of samples. The presence of contaminating protein or phenol, which absorb at 280 nm, decrease the ratio. RNA preparations with an A_{260} /A_{280nm} of 1,8 to 2,0 indicated that the samples were pure. An example of a spectrophotometric scan is given in figure 8.

4.2.3 Gel electrophoresis

All the RNA samples obtained by the extraction procedures described in chapter 4.2.1 were electrophoresed in a denaturing formaldehyde agarose gel according to Maniatis and co-workers (1982). A 1,5% agarose minigel was prepared by dissolving the agarose in water by heating in

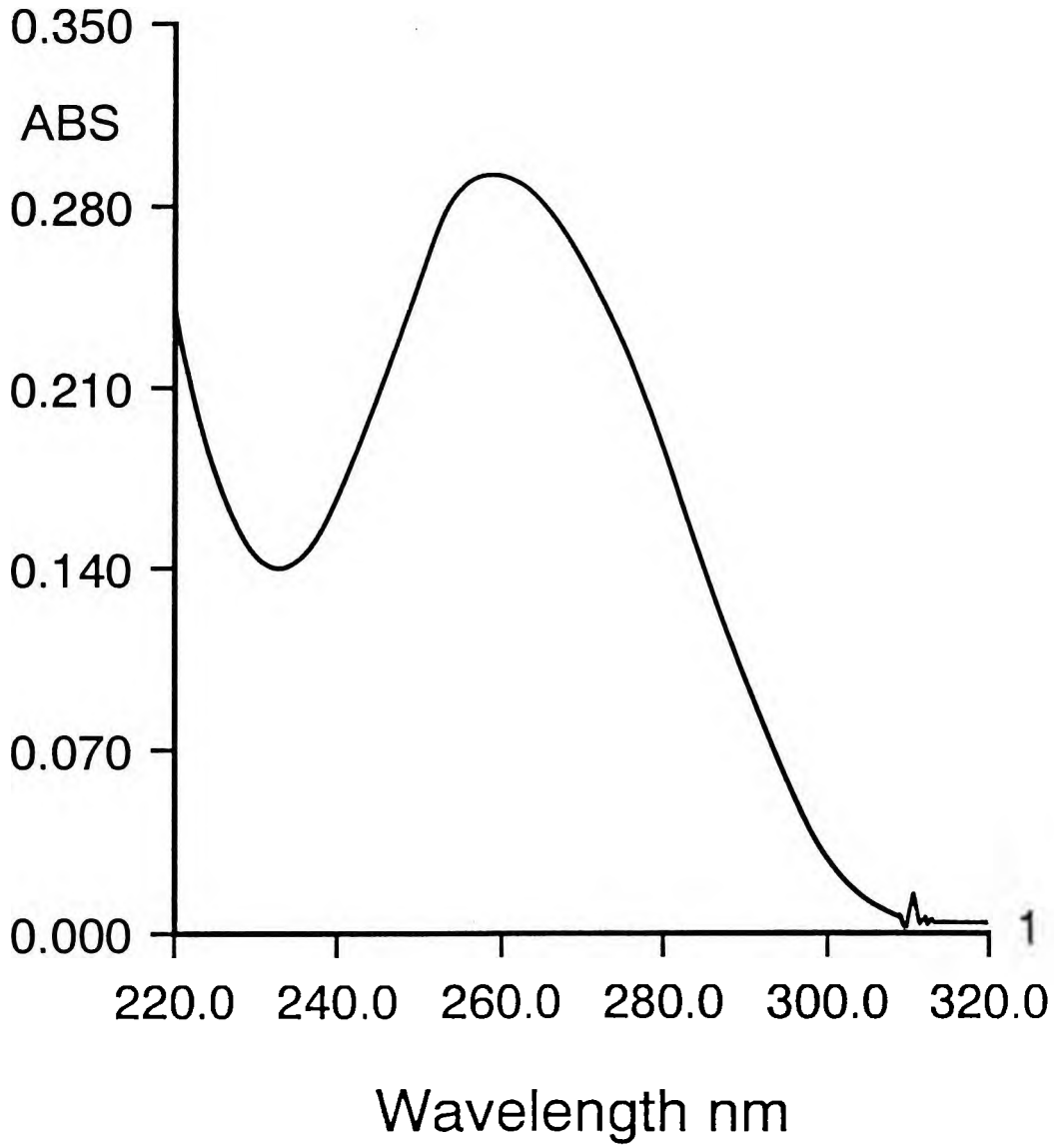

Figure 8: RNA extracted from 8 day cultured macrophages was assessed by spectrophotometric scanning in the range of 220 to 320 nm.

a microwave oven. The solution was allowed to cool to 50 to 60°C before adding formaldehyde to a 2,2 M final concentration. The agarose solution was poured into a gel-mold and well-forming combs were positioned. After setting, the gel was submerged in an electrophoresis apparatus containing MOPS running buffer (morpholino-propanesulfonic acid 0,2 M pH 7,0; sodium acetate 50 mM; EDTA 1mM). The combs were then carefully removed. Before electrophoresis, 2 µl of the RNA solution (containing 2 to 6 µg) was denatured at 55°C for 15 minutes in 14,5 µl of sample buffer (running buffer 1 µl; deionized formamide 10 µl; formaldehyde 3,5 µl). 2 µl of loading buffer (glycerol 50% ; EDTA 1 mM; bromophenol blue 0,4%; xylene cyanol 0,4%) and 1 µl ethidium bromide (1 mg/ml) was added to each Eppendorf tube. The samples were carefully applied to individual wells and the gel was run at 75V for approximately 3 to 4 hours. The ethidium bromide stained bands were photographed on an ultraviolet trans-illuminator. The integrity of the RNA was assessed by evaluation of the 28S and 18S ribosomal RNA (rRNA) bands and a ratio of 2 : 1 excluded degradation of the nucleic acid. Figure 9 represents the electrophoretic pattern of total RNA isolated from cultured macrophages by the guanidinium thiocyanate-phenol-chloroform method. A similar pattern was observed when RNA was extracted by the lithium chloride method (figure 10). The RNA was separated according to its molecular weight and the presence of 28S and 18S rRNA confirmed that the RNA was intact.

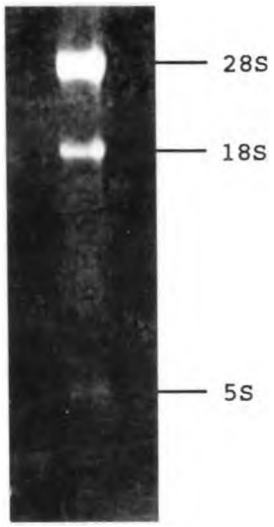

Figure 9: Agarose gel electrophoretic pattern of intact total RNA extracted from cultured macrophages by the guanidinium thiocyanate-phenol-chloroform method.

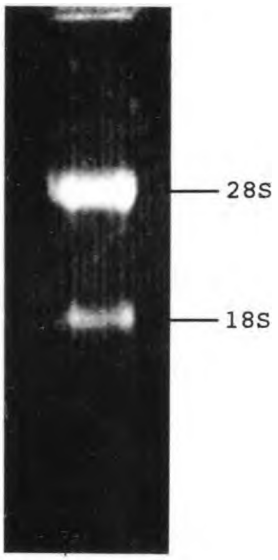

Figure 10: RNA extracted from cultured macrophages by the lithium chloride method was separated by gel electrophoresis. Intact 28S and 18S rRNA bands are shown.

A comparison of the electrophoretic analysis of cytoplasmic RNA recovered by the method of Gough (1988) and the guanidinium thiocyanate technique is depicted in figure 11. Using the former method, the results show degraded RNA as detected by the absence of visible bands and a smear down the gel track. The latter method demonstrates the presence of undegraded 28S and 18S rRNA bands, which are 5,1 kb and 2,0 kb respectively, and can be used as markers (Davis et al, 1986).

4.2.4 RNA blotting

Northern blotting is a technique whereby electrophoretically resolved RNA is transferred from a gel matrix to a solid support for the purpose of analyzing specific sequences with radiolabelled probes by hybridization. Methods have been developed for the transfer of RNA from denaturing gels onto nitrocellulose or nylon membranes (Thomas, 1980; Meinkoth and Wahl, 1984). RNA preparations can also be directly immobilized onto these membranes by a process of 'slot-blotting.' However, a prerequisite for this technique is the presence of intact RNA which has to be confirmed by electrophoresis of the nucleic acid. In the current study, RNA was either transferred from an agarose gel to Hybond-N (nylon) membrane (Amersham, UK) or directly applied to slots on the membrane. Details of the procedures were followed according to the manufacturers recommendations and with the aid of the manual by Davis and colleagues (1986).

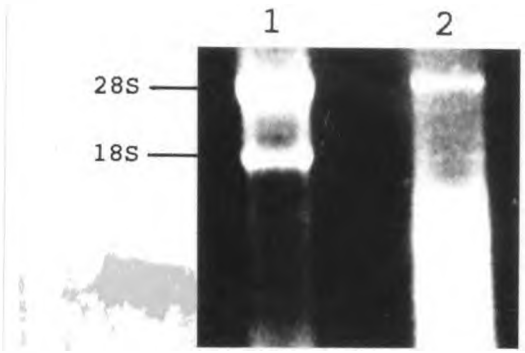

Figure 11: Ethidium stained agarose gel showing extracted RNA from cultured macrophages by the guanidinium thiocyanate technique (lane 1). RNA extracted by the NP40-urea-SDS method (lane 2) was significantly degraded as evidenced by a smear down the gel track.

Following electrophoresis, the agarose minigel was rinsed twice in 10 x SSC (sodium chloride 1,5 M; sodium citrate 0,15 M) for 20 minutes to remove excess formaldehyde. The gel was placed, with well openings facing down, on a glass plate covered with Parafilm plastic. The Hybond-N membrane was laid over the gel and any air bubbles were removed by rolling a glass stirring rod over the surface. Two sheets of filter paper (Whatman 3 MM) were placed on top followed by a 5 to 7 cm stack of paper towels, a glass plate and a 1 kilogram weight to compress the layers. Transfer of the RNA was complete after 12 to 15 hours. The transfer efficiency could be checked by staining the gel in ethidium bromide (1 μg/ml) in water. The blot was rinsed in 2 x SSC and allowed to air dry. It was then covered with cling wrap and exposed to uv light (RNA side down) for 5 minutes to ensure covalent retention of the RNA. The blots were stored at 4°C ready for hybridization.

In the next procedure, RNA samples were directly applied onto Hybond nylon membrane with the aid of a slot blot apparatus. The RNA was denatured by incubating 2 to 5 μg quantities in 20 x SSC (to 50 μl) and 50 μl deionized formamide at 55°C for 15 minutes. Two pieces of Whatman 3MM paper were first placed in the apparatus and the nylon membrane was placed on top. Both were presoaked in 20xSSC. A vacuum source was attached and each slot was rinsed with 100 μl 20 x SSC. The pretreated RNA samples were added to the slots and each well was rinsed with 100 μl 20 x SSC. The blot was removed, air dried and placed on an ultraviolet transilluminator for 5 minutes and then kept at 4°C.

4.3 cDNA PROBES

Single-stranded complimentary DNA (cDNA), possessing information contained in a specific mRNA species, is constructed by using the enzyme reverse transcriptase. The single-stranded DNA is then used to direct the synthesis of a complimentary DNA strand. The cDNA sequence is then cloned into a vector DNA molecule to provide unlimited material for further use. Plasmids, which are small circular extrachromosomal molecules, are commonly used as carriers and possess genes for properties such as antibiotic resistance. This feature can be used as a selectable marker for the presence of the cDNA-containing plasmid. For the purpose of this study, three cDNA clones were used to detect the presence of specific mRNA molecules under investigation.

4.3.1 Human ß-actin

A human ß-actin cDNA, pHF ß-AI, was kindly provided by Dr. I. Parker, University of Cape Town with permission from Dr. L. Kedes, Veterans Administration Medical Centre, California. This full length cDNA is inserted in the Okayama-Berg cloning expression vector (Gunning et al, 1983). The plasmid was obtained in E. coli culture and maintained by selection with ampicillin. The insert was released with Bam HI as a 1,9-2,1 kb fragment (figure 14).

4.3.2 Human H-ferritin

The clone for the ferritin H chain has been isolated from a human liver cDNA library by screening with a H cDNA clone from a human lymphocyte cDNA library (Boyd et al, 1984 & 1985). The clone, pHF 16, was provided by Dr. J.W. Drysdale, Tufts University School of Medicine, Boston, Massachusetts. The H cDNA clone contains a 77-base 5' noncoding region, a 552 open reading frame and a 161-base 3' noncoding region. The cDNA is inserted in the Pst I restriction site of the plasmid vector pKT 218 with a 530-base deletion (Talmadge et al, 1980). The plasmid was obtained in E. coli culture and maintained by tetracycline selection. The restriction map is shown in figure 12. The cloned DNA was cleaved from the plasmid by digestion with Pst I and Rsa I (figure 15). The 430-base H cDNA fragment was used to detect specific mRNAs.

4.3.3 Human L-ferritin

The clone, pLF 108, was obtained from Dr. J.W. Drysdale. The cDNA clone that contains the entire coding region of L ferritin was isolated from a human liver cDNA library by screening with oligonucleotides (Boyd et al, 1985). It contains a 151-base 5' noncoding region, a 528 base coding and a 143-base 3' noncoding region. The insert is also situated in the Pst I restriction site of pKT 218 and was maintained by tetracycline selection in E. coli culture. The restriction map is shown in figure 13.

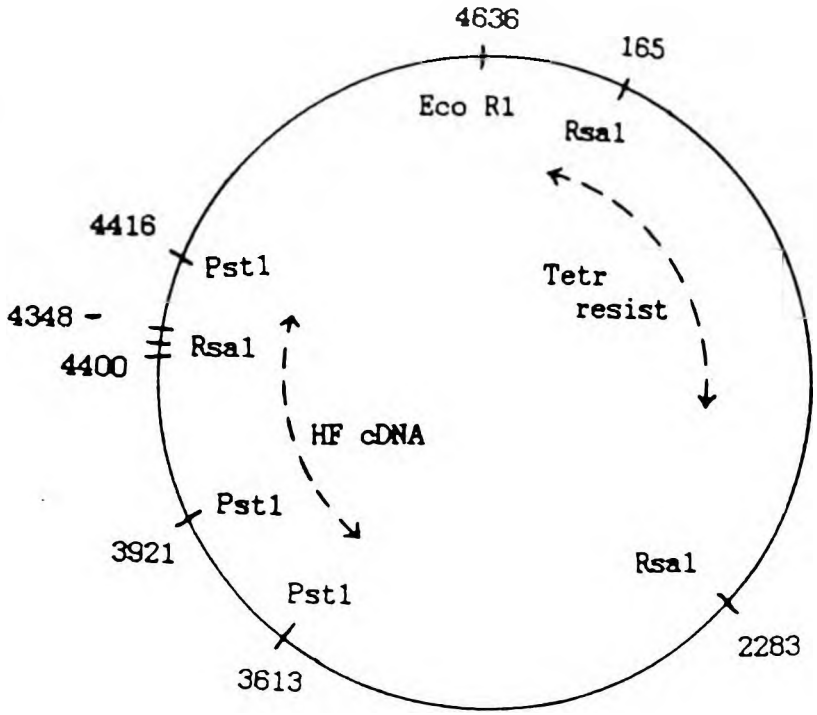

Figure 12: Partial restriction map of the human H-ferritin cDNA containing plasmid, pHF 16.

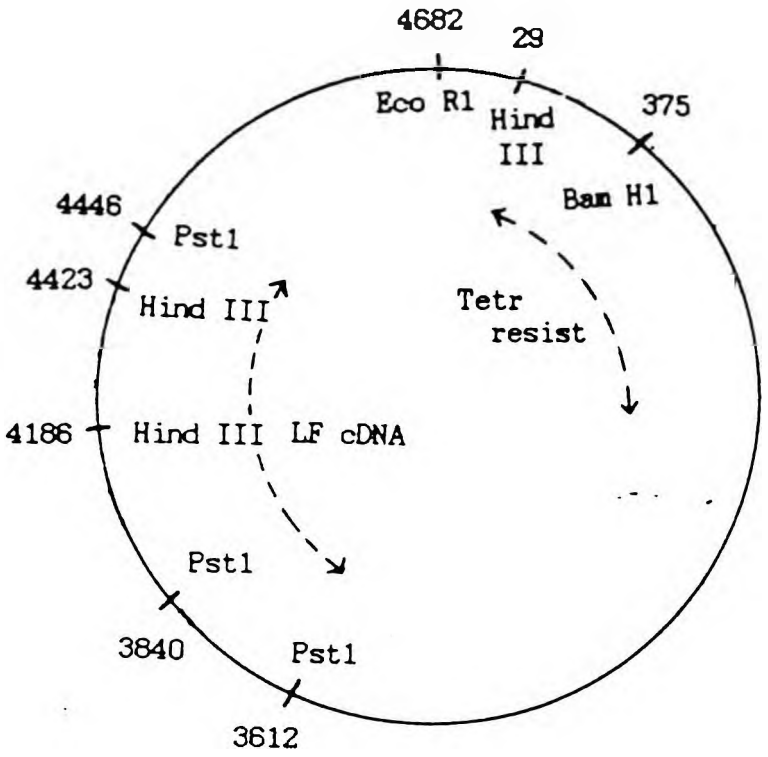

Figure 13: Partial restriction map of the human L-ferritin cDNA containing plasmid, pLF 108.

The insert was released by digestion with the enzymes, Pst I and Hind III, and the electrophoretic pattern is shown in figure 16. The 224-base fragment was used for probing RNA.

4.4 PREPARATION OF PLASMID DNA

4.4.1 Growth and maintenance of bacteria

The ß-actin and human H- and L-ferritin cDNA-containing plasmids were obtained in E. coli cultures. The plasmid vectors contain a gene that encodes a protein that confers antibiotic resistance so that E. coli host cells can be grown on selective medium. The coli borne inserts were either grown in liquid or on agar plates. For liquid culture, the usual medium was Luria-Bertani (LB) medium (tryptone 1%; yeast extract 0,5%; NaCl 1%). For solid medium, 1,5% of bacteriological agar was added. The medium was autoclaved at 120°C for 20 minutes and allowed to cool to 60°C. The appropriate antibiotics were added (50 μ/ml ampicillin for ß-actin and 12,5 μ/ml tetracycline for ferritin-subunits) and aliquots were poured onto petri dishes for the solid medium. The coli-containing agar plates were tightly wrapped in parafilm plastic and stored at 4°C for a couple of weeks. For long-term storage, 0,6 ml of sterile 50% glycerol solution was added to 1,5 ml aliquots of the bacterial culture grown overnight in LB medium and stored at -70°C in sterile screw-capped dram bottles.

4.4.2 Isolation and purification techniques

Plasmid DNA was isolated and purified in order to provide probe DNA for the hybridization procedures. Preparative quantities of the plasmid DNA were extracted by alkaline lysis (Maniatis *et al.* 1982) and further purification was achieved by polyethylene glycol precipitation.

Plasmid-containing *E. coli* cells were grown up in 500 ml of LB medium containing the appropriate antibiotics. The cells were spun down at 5000 *g* for 10 minutes and the supernatant discarded. The cell pellet was washed by resuspending in 100 ml ice-cold TSE (Tris-HCL 10 mM, pH 7,8; EDTA 1mM; NaCl 100mM) and centrifuged again. The pellet was then resuspended in 10 ml ice-cold TGE (Tris-HCL 25 mM, pH 8; glucose 50mM; EDTA 10 mM) and lysozyme was added to 5 mg/ml in order to digest the proteoglycan layer of the cell wall. The cell suspension was thoroughly mixed and left to stand at room temperature for 10 minutes. 20 ml of freshly prepared alkaline NS solution (sodium hydroxide 200 mM; sodium dodecyl sulphate 1%) was added and mixed by gently inverting several times and then incubating on ice for 10 minutes. During this process, the protoplasts are lysed and total cellular DNA is released. The chromosomal DNA is denatured, whereas the plasmid DNA is only partially denatured due to its circular form. The pH was lowered to approximately 5,0 with 15 ml ice-cold potassium acetate (potassium 3 M; acetate 5 M) followed by incubation on ice for 10 minutes. This step causes the

chromosomal DNA to renature randomly producing an insoluble protein-DNA-SDS and high molecular weight RNA precipitate. The plasmid DNA renatures as well but both strands complement properly keeping the plasmid soluble. The cell debris and chromosomal DNA were pelleted by centrifugation at 76 000 g for 20 minutes at 4°C, leaving a supernatant containing predominantly plasmid DNA and low molecular weight RNA. The plasmid was further purified by polyethylene glycol precipitation. Contaminant RNA was removed by the addition of RNAase A to a final concentration of 20 μ/ml and incubated at 37°C for 20 minutes. To remove protein, an equal volume of phenol-chloroform (redistilled phenol + 0,1% 8-hydroxy-quinoline equilibrated with 0,1 M Tris pH 8 and 0,2% ß-mercapto-ethanol; chloroform:isoamylalcohol 24:1) was added and incubated on ice for 10 minutes. The phases were separated by centrifugation at 5000 g for 10 minutes. The upper aqueous layer was collected and to it was added 1/10 of 1 volume of 3 M sodium acetate, pH 7. The plasmid DNA was recovered by the addition of 0,6 volumes of isopropanol at -70°C and then centrifuged at 12 000 g for 30 minutes. The pellet was washed in 70% ethanol and after recentrifugation, it was dried in a vacuum centrifuge. The DNA precipitate was dissolved in 1,6 ml water and 0,4 ml NaCl (4M) and 2 ml of polyethylene glycol (13%) were added. The solution was allowed to stand on ice for 10 minutes and after centrifugation at 10 000 g, the resultant pellet was washed in ethanol, dried and resuspended in TE buffer (Tris-HCL 10mM

pH 8; EDTA 1mM). All samples were stored at -20°C. The plasmid DNA was quantitated by optical density measurement at 260nm and the purity assessed by recording the reading at 280nm. A ratio of 1,8 to 1,9 (A_{260} / A_{280}) indicated that the samples were devoid of protein and phenol. Alternatively, the yield and purity of the plasmid DNA was determined by running an aliquot on an agarose minigel alongside a series of standard DNA solutions.

4.4.3 Restriction enzyme digestion

The cDNA inserts were released from the plasmids by making use of specific restriction endonuclease (RE) enzymes in the presence of appropriate buffers. These were obtained from Boehringer Mannheim, Mannheim, Germany. As discussed in chapter 4.3., pHF ß-AI was digested with Bam HI; the cDNA was released from pLF 108 using Pst I and Hind III and a double digest with Pst I and Rsa I was performed on pHF 16.

The plasmid DNA (1-10μg) was mixed with water to give a volume of 18 μl in 1,5 ml Eppendorf tubes. Two μl of the appropriate buffer and 10-20 units of restriction enzyme were added as follows:

For pHF ß-AI : Enzyme Bam HI in buffer B

For pHF 16 : Rsa I in buffer L followed by Pst I with the addition of 1 μl NaCl (2M)

For pLF 108 : Hind III in buffer M followed by Pst I in buffer B.

After incubation at 37°C for 1 hour, the reaction was terminated by the addition of 0,5 μl EDTA (0,5M). The samples were then denatured at 65°C for 5 minutes followed by rapid cooling on ice. Two μl of loading buffer (glycerol 50% w/v; EDTA 1mM; bromophenol blue 0,4% w/v; xylene cyanol 0,4% w/v) was added to each tube.

4.4.4 Restriction product gel electrophoresis

The products of the endonuclease digestion were resolved by analytical gel electrophoresis in agar. For the ß-actin and ferritin-subunit probes 0.9% and 1,6% agarose minigels were prepared respectively. Low melting point agarose (Bio-Rad, California) was dissolved in distilled water by heating in a microwave oven. After cooling to 60°C, an appropriate amount of TBE buffer (Tris-HCL 0.089 M pH 8,0; boric acid 0.089 M; EDTA 2 mM) and ethidium bromide (to a final concentration of 0,5 μg/ml) was added. The solution was poured into a mold with gel combs in position and allowed to set. After loading of the DNA samples, the gel was electrophoresed in TBE running buffer at 50V for 3 to 4 hours at 4°C. The samples were run alongside molecular weight markers III (Boehringer Mannheim, Germany) prepared in the same way as before. The gels were photographed on an ultraviolet light source and the results are depicted in figures 14, 15 and 16.

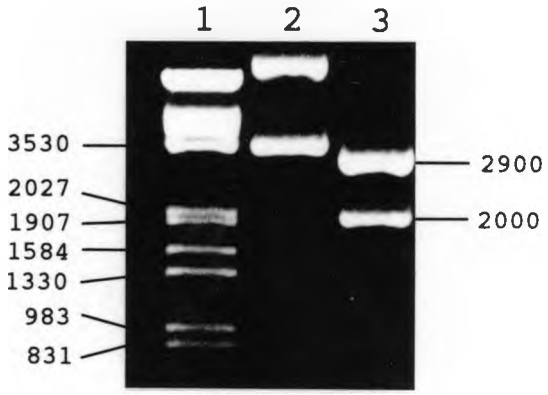

Figure 14: Agarose gel electrophoresis representing 2900 bp and 2000 bp fragments after BAM HI digestion of plasmid DNA from pHF ß-AI (lane 3). Lane 1 represents molecular weight size markers and lane 2 undigested plasmid DNA.

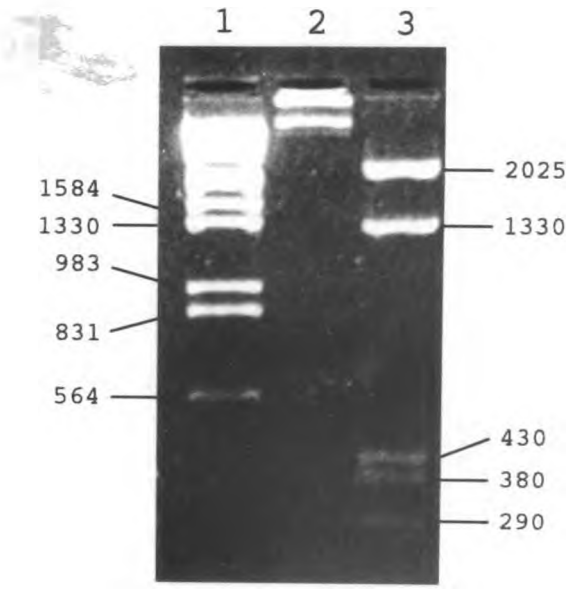

Figure 15: Agarose gel electrophoresis of DNA fragments after PST I and RSA I restriction digestion of pHF 16 (lane 3). Lane 1 represents molecular weight size markers and lane 2 undigested plasmid DNA.

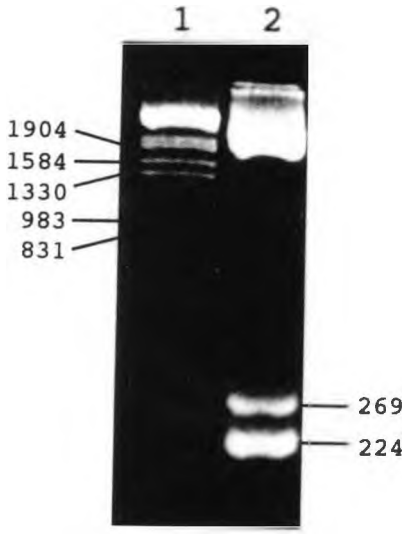

Figure 16: Agarose gel electrophoresis of DNA fragments after PST I and HIND III digestion of pLF 108 (lane 2). Lane 1 represents molecular weight size markers.

For recovery of the DNA fragments from the agarose, the desired cDNA bands were excised and dissolved in 400 µl of distilled water at 65°C for 15 minutes. To 200 µl aliquots was added 200 µl of phenol in TE buffer and 200 µl of chloroform:isoamylalcohol (24:1). After mixing the contents, the tubes were spun at 10 000 g for 20 minutes to separate the phases. The upper aqueous layer was carefully removed and to it was added NaCl to a final concentration of 100 mM. The DNA was concentrated by adding 1 volume of isopropanol and chilled to -20°C overnight. Each sample was centrifuged at 10 000 g for 20 minutes at 4°C and the resultant pellets washed in 70% ethanol. The tubes were recentrifuged and traces of ethanol removed by vacuum drying. The DNA pellet was dissolved in 30-50 µl of TE buffer and the amount of nucleic acid was quantitated according to Maniatis and colleagues (1982). 1-5 µl of sample was spotted onto parafilm wrap over a UV source. Appropriate concentrations of control DNA were also spotted and 1 µl of TE solution containing 2 µl of ethidium bromide was added to each spot. The amount of DNA was estimated by comparing the fluorescence emitted with that of the standards.

To confirm that the correct cDNA fragments were purified, a sample of each probe was electrophoresed with size markers in agarose. Figure 17 demonstrates the ß-actin band (2,0 kb) run on a 0,9% gel and figure 18 shows the H-ferritin (430 bp) and L-ferritin (224 bp) fragments run on 1,6% agarose gels.

Figure 17: Ethidium stained 0,9% agarose gel demonstrating the 2,0 kb purified ß-actin band (lane 2). Lane 1 represents molecular weight size markers.

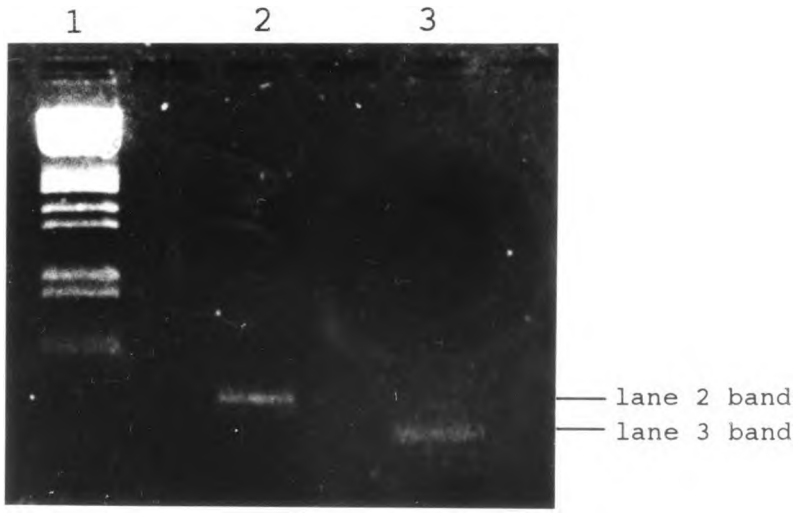

Figure 18: Ethidium stained 1,6% agarose gel of the 430 bp purified H-ferritin fragment (lane 2) and 224 bp L-ferritin fragment (lane 3). Lane 1 represents molecular weight size markers.

4.5 LABELLING PLASMID-DERIVED PROBES

The DNA restriction endonuclease fragments were radiolabelled according to a random primer method developed by Feinberg and Vogelstein (1983). In this method hexanucleotides of a random sequence are used to prime DNA synthesis on denatured template DNA. The Klenow fragment of DNA polymerase I is used to synthesize a new complementary DNA strand from the 3'-hydroxyl termini of the hexamer primer. ^{32}P-labelled and unlabelled deoxy-nucleoside triphosphates present in the reaction are incorporated into the newly synthesized complementary DNA strand. A commercially available kit obtained from Boehringer Mannheim (Mannheim, Germany) was used.

The cDNA to be labelled (1 to 9 μl, 20-200 ng in TE buffer) was denatured by heating to 100°C for 2 minutes in a boiling water bath and then chilled on ice. To this was added an appropriate volume of each reagent in the following order: 3 μl of nucleotide mix (dATP, dGTP, dTTP); 2 μl of reaction mixture (random hexamers in reaction buffer); 5 μl of α-^{32}P dCTP (50 μCi at 3000 Ci/mmol, Amersham, UK) and 1 μl of Klenow enzyme. The contents were incubated at 37°C for 30 to 90 minutes. The reaction was terminated by the addition of 2 μl 2,3 mol/l EDTA (pH 8) and/or by heating to 65°C for 10 minutes.

The efficiency of labelling was monitored by determining the percentage incorporation of radiolabel into the newly synthesized DNA. The most commonly used technique involves the incorporation of radioactivity into

acid-insoluble material by trichloroacetic acid (TCA) precipitation. For this purpose, 1 μl of reaction mixture was diluted into 100 to 200 μl of water containing 50 μl carrier DNA (at 1 mg/ml). 50 μl of this sample was spotted onto Whatman GFC glass microfibre filter discs contained in scintillation vials. This gave the total number of counts in the sample. To an equal volume of the sample was added 2 ml ice-cold 10% TCA. The mixture was vortexed and kept on ice for 10 to 15 minutes to allow co-precipitation of the labelled and carrier DNA. The precipitated DNA was collected on glass microfibre filter discs by vacuum filtration and then washed well with 10% TCA. All the filter discs were dried thoroughly and after the addition of 10 ml scintillation fluid to each, the radioactivity was determined by liquid scintillation counting. The percentage incorporation was calculated as follows:

$$\% \text{ incorporation} = \frac{\text{precipitated counts}}{\text{total counts}} \times 100$$

The amount of newly synthesized DNA (ng) was determined according to the formula:

$$\frac{\text{total number of } \mu\text{Ci added} \times 13.2 \times \% \text{ incorporation}}{\text{specific activity of dCTP (Ci/mmol)}}$$

$$\frac{\mu\text{Ci dCTP } (50\mu\text{Ci}) \times 13.3 \times \% \text{ incorporation}}{\text{specific activity of dCTP (3000 Ci/mmol)}}$$

The specific activity in dpm/μg was determined as follows:

$$\frac{\text{incorporated radioactivity in dpm}}{\text{input DNA + newly synthesized DNA}}$$

$$\frac{\mu\text{Ci dCTP } (50\mu\text{Ci}) \times 2.2\times10^{4} \times \% \text{ incorporation} \times 10^{3}}{\text{input DNA + newly synthesized DNA}}$$

A specific activity of 0,7 x 10^9 to 1,8 x 10^9 dpm/μg was achieved corresponding to 71% incorporation and 65% incorporation respectively. The removal of unincorporated label by gel filtration or selective precipitation was found not to be necessary as the incorporation exceeded 60%.

4.6 HYBRIDIZATION

4.6.1 Introduction

Molecular hybridization is the formation of double-stranded nucleic acid molecules by sequence specific base pairing of complementary single strands (Britten and Davidson, 1985). Electrophoretically separated RNA species that are transferred to solid supports (blots) are amenable to this technique. A radiolabelled cDNA probe is then capable of hybridizing to complementary sequences found among the molecules bound to the solid support. The association and dissociation kinetics governing complementary hydrogen bonding between the base pairs of nucleic acids are influenced by many factors. Among the factors influencing this equilibrium are concentration, temperature and salt concentration of the hybridization buffer and the presence of rate enhancers, helix destabilizers and blocking agents. The quality of the results is further affected by probe related variables such as probe specific activity, probe concentration and probe length.

In this section, northern blots and slot blots containing transferred RNA were hybridized by modification

of a protocol described by the manufacturers of Hybond N membrane (Amersham, UK) followed by quantitative auto-radiography to identify and measure the amount of RNA species of interest.

4.6.2 Conditions for hybridization

The hybridization procedure can be divided into 3 steps : prehybridization, hybridization with labelled probe and washing. In the prehybridization step the blots are incubated in a solution which pre-coats all the sites which would bind the probe non-specifically. The northern blots and slot blots were prehybridized in heat seal bags at 42°C for 4 to 5 hours in a constant shaking water bath. 5 to 20 ml of prehybridization solution was used for each bag. The solution contained the following : 6,25 ml 20x SSPE (3,6 M NaCl; 0,2 M sodium phosphate, pH 7,7; 0,02 M EDTA); 12,5 ml deionized formamide; 1,25 ml 100x Denhardts solution (2% bovine serum albumin [w/v], 2% Ficoll [w/v], 2% polyvinyl pyrollidone [w/v]); 3,75 ml water; 200 µl (2mg) denatured herring sperm DNA and 1,25 ml 10% (w/v) sodium dodecyl sulphate (SDS).

Following incubation, the prehybridization buffer was removed and replaced with 5 to 15 ml (per bag) of hybridization buffer containing the denatured labelled cDNA probe. 20 µl of the labelled reaction mixture was diluted to 50 µl with water and then denatured at 100°C for 2 minutes and chilled on ice. An aliquot of 10 µl was added to each northern blot and 10 to 15 µl to each slot blot.

The hybridization solution was made up by adding in the following order : 6,25 ml 20x SSPE; 12,5 ml deionized formamide; 1,25 ml Denhardts solution; 2 ml water; denatured labelled cDNA probe and 4% (w/v) polyethylene glycol (MW 8000). The blots were hybridized in a shaking water bath at 42°C for at least 12 hours.

Following hybridization the blots were washed. For the detection of H- and L-ferritin mRNA species in human macrophages using ferritin cDNA probes, the blots were washed as follows: In the first wash, the blots were incubated in 2x SSPE and 0,1% (w/v) SDS at 42°C for 15 minutes. The procedure was repeated once. The solution was replaced with 1x SSPE and 0,1% (w/v) SDS and incubated at 42°C for 30 minutes. This was followed by a high stringency wash in 0,1x SSC (15 mM NaCl; 1,5 mM sodium citrate) with 0,1% (w/v) SDS at room temperature for 15 minutes.

When human macrophage RNA was probed with the human ß-actin probe, the washes were carried out in the following order:

WASH 1: Each blot was washed briefly at 42°C in 2x SSC (0,3 M NaCl; 0,03 M sodium citrate) ;

WASH 2: 2x SSC with 0,1% SDS for 10 minutes at room temperature ;

WASH 3: Repeat wash 2 ;

WASH 4: 1x SSC with 0,1% SDS at 65°C for 15 minutes ;

WASH 5: 0,1x SSC with 0,1% SDS at 65°C for 10 minutes.

The blots were air dried and wrapped in cling film. The location of radioactivity on the blots was detected by autoradiography. The blots were placed in the dark under preflashed Kodak XAR-5 film enclosed in an X-ray film cassette with intensifying screens. The use of intensifying screens and preflashed film ensures maximum sensitivity. The film was exposed at -70°C for 36 to 48 hours and developed in an automated developer. Figure 19 demonstrates autoradiographs of northern blots of RNA extracted from 8 day cultured macrophages that were probed with human H- and L-ferritin and ß-actin probes.

4.6.3 Quantitation of autoradiographs

The amount of radioactivity bound to the slot blots was quantitated by densitometric scanning of the autoradiographs using a video densitometer and image analyzer (Biomed Instruments, Fullerton, California).

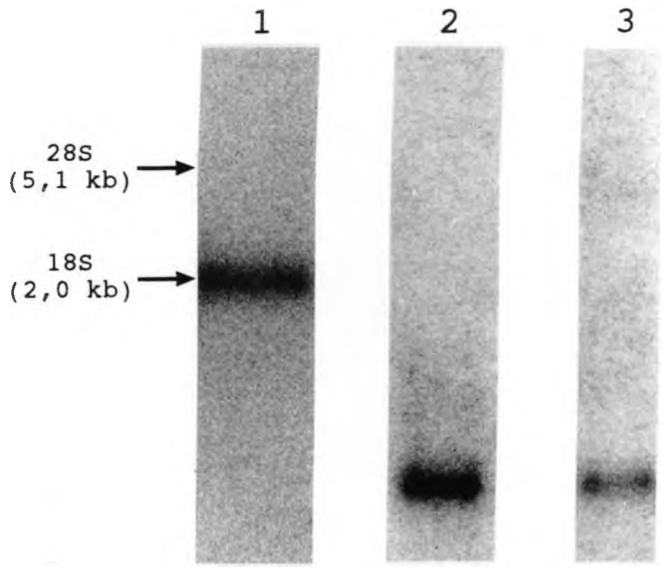

Figure 19: Autoradiogaphs of northern blots of human macrophage RNA probed for ß-actin (lane 1), H-ferritin (lane 2) and L-ferritin (lane 3) confirming probe specificity. Relative positions of 28S and 18S rRNA are indicated by arrowheads.

SECTION 3

CHAPTER 5

FERRITIN MESSENGER RNA IN MACROPHAGES

5.1 INTRODUCTION

The observation that the reticuloendothelial cells contain little iron in HLA-linked haemochromatosis has led many investigators to believe that an abnormality in ferritin metabolism may be responsible, leading to reduced iron storage within these cells and increased delivery of iron to the plasma. The introduction to this chapter focuses mainly on the regulation of ferritin messenger RNA (mRNA) in relation to iron and ultimately the synthesis of ferritin in the setting of the RES.

Cells of the mononuclear-phagocyte system play a pivotal role in the storage of iron and have been shown to contain significant amounts of ferritin (Summers et al, 1975). Variations in the proportion of H- to L- subunits within these cells have been reported. In normal RE iron metabolism, it is generally supposed that macrophages accumulate L-rich isoferritins (Harrison, 1986; Worrall and Worwood, 1991; Raha-Chowdhury et al, 1993) to accomodate larger amounts of iron for long term iron storage (Bomford et al, 1978; Theil, 1987). In contrast, monocytes are relatively rich in H-type subunits (Powell et al, 1975a; Jones et al, 1983) and it has been suggested that they are involved in recycling of iron to the intermediate labile iron pool for metabolic processes (Jacobs, 1985; Pattanapanyasat, 1989). Worrall and Worwood (1991) were not able to demonstrate a difference in the increase of H- and L-type ferritin protein subunits in response to iron between normal subjects and haemochromatotic patients.

Ferritin synthesis can be regulated either at a transcriptional or a translational level or both [section 1, chapter 1.3.6]. The characteristics of ferritin mRNA during the in vitro maturation of human monocytes to macrophages were investigated by Testa and co-workers (1989). The authors documented a considerable increase in mRNA for both H and L ferritin during monocyte incubation in vitro for 8 days. However, the addition of iron as ferric ammonium citrate to the cultures stimulated ferritin synthesis but only slightly enhanced H- and L-ferritin mRNA levels. The effect observed during the in vitro maturation of monocytes could be related to transcriptional activation whereas upon iron stimulation a translational mechanism is mainly involved.

It is clear that the IRE and IRP play an important role in the maintenance of intracellular iron homeostasis by coordinately regulating the translation of the mRNAs for ferritin and the transferrin receptor [section 1, chapter 1.3.6.2]. This mechanism has recently been shown to be intact in the gut in haemochromatosis (Pietrangelo et al, 1993). The distribution of iron in gut and RE cells in haemochromatosis (Brink et al, 1976) suggests that these cells share common intracellular iron mechanisms, at least with respect to ferritin.

With regard to overall ferritin synthesis in RE cells in haemochromatosis, Saab and colleagues (1986), observed that monocyte ferritin content was decreased in patients with haemochromatosis. However, a number of investigators

have revealed no differences in ferritin synthesis and ferritin content in monocytes between patients and normal subjects (Jacobs and Summers, 1981; Bassett et al. 1982). It should be noted that these studies were conducted mainly on monocytes and therefore may not be an accurate reflection of ferritin metabolism in macrophages. Recently, Gordeuk and co-workers (1992) showed that cultured monocytes from haemochromatotic patients released decreased amounts of TNF-α compared with normal cells. The author suggested that ferritin synthesis may in fact be impaired within cells of the mononuclear phagocyte system of haemochromatotics, either by decreased transcription of mRNA or reduced translational activity. It is more likely that the TNF-α gene located close to the HLA locus on chromosome 6 (Spies et al. 1986), the gene for hereditary haemochromatosis, may play a role in the pathogenesis of the disease rather than the postulation that a mutation in the IRP (Kuhn, 1991), coded on chromosome 9 (Hentze et al. 1989a), could be related to the dysfunction in iron metabolism in haemochromatosis.

It is apparent that many factors affecting ferritin biosynthesis have to be considered carefully when devising a suitable model of iron metabolism. With regard to mononuclear phagocytes, the type of cell (monocyte or macrophage), metabolic state and iron status and source of iron are amongst the factors that appear to be important.

The current study aimed to examine the hypothesis that an abnormality in reticuloendothelial ferritin

synthesis exists in haemochromatosis. To this end, the levels of ferritin mRNA in response to iron were investigated in normal and haemochromatotic macrophages cultured in vitro. The methodologies described in chapters 2.2 and 4 were applied to this study.

5.2 SUBJECTS AND METHODS

300 ml of blood was collected into citrate from healthy adult volunteers (with informed consent) and during therapeutic venesection from patients with haemochromatosis. The patients, who had excess iron removed previously, were only bled occasionally during monitoring.

Mononuclear cells were separated by centrifugation over Ficoll-hypaque followed by adherence to tissue culture petri dishes according to methods described in section 2, chapter 2.2. Each 35mm diameter petri dish contained 3 x 10^6 cells in 2,5 ml BMESS with 10% autologous serum and antibiotics. The cells were incubated at 37°C in a 5% CO_2 incubator for 8 days. Cell viability ranged from 85 to 100% as determined by the trypan blue exclusion test. The adherent cells were characterized and more than 90% demonstrated non-specific esterase activity and phagocytosed yeast particles. All experiments were performed on the eighth day of culture as follows :
After the non-adherent cell population and culture medium were decanted, the petri dishes were washed 3 times with PBS. Ten petri dishes were set up for each time interval

to be studied. To each dish was added 2 ml RPMI and 10% fetal calf serum and the cells were preincubated for 1 hour at 37°C. Ferric ammonium citrate was added to each culture dish to give a final concentration of added iron of 200 μM. The fetal calf serum contributed an additional 3 μM of iron. Ferric ammonium citrate has been shown to enhance ferritin synthesis (Bassett et al. 1982) and ferritin mRNA (Testa et al. 1989) in both normal and haemochromatotic cultured monocytes. Control dishes containing cells and RPMI medium but no added iron were also included. The cells were incubated at 37°C under 5% CO_2 and 95% humidity for various periods up to 8 hours.

At 0, 4, 6, and 8 hours after incubation, the medium was removed and each dish was washed 3 times with PBS. Total RNA was prepared by extraction from the macrophages at various times by the guanidinium isothiocyanate phenol-chloroform technique [chapter 4.2.1]. The contents of 10 dishes (for each time interval) were pooled into 1 Eppendorf tube and each RNA specimen was assessed spectrophotometrically. The RNA integrity was evaluated by electrophoresis in 1,5% formaldehyde agarose gels. For slot blot analysis, 5 μg of RNA were added to each slot and the blots were hybridized with ^{32}P-labelled human H- and L-ferritin subunit probes and the human ß-actin probe. After washing, the blots were autoradiographed at -70°C for 48 hours. For northern blot analysis (to confirm probe specificity), 5 μg of total RNA was run on 1,5% formaldehyde gels, transferred onto Hybond nylon membrane

by northern blotting, hybridized and exposed as for the slot blots. The intensity of radioactive bands on the slot blots was analyzed by densitometric scanning using a video densitometer and image analyzer (Biomed, California, USA). The amount of mRNA was quantitated by determining the areas under the peaks obtained for each slot. The control (0 hour) time points in each experiment were normalized to 1. The final data were expressed as the relative amounts of H- and L- ferritin mRNA to the constitutively expressed ß-actin mRNA levels.

5.3 RESULTS

To evaluate the effect of iron on ferritin mRNA levels, 8 day cultured macrophages from normal subjects and haemochromatotic patients were incubated in the presence of ferric ammonium citrate for up to 8 hours. Autoradiographs of representative slot blots containing RNA probed with ß-actin (used as a constitutively expressed control), H-ferritin and L-ferritin subunit probes from a normal subject and a haemochromatotic patient are shown in figures 20, 21 and 22, respectively. In each figure autoradiographs of northern blots demonstrate integrity of the corresponding message.

The slot blots for each patient and probe used were scanned quantitatively by densitometry and the resultant peaks are depicted in figures 23 and 24.

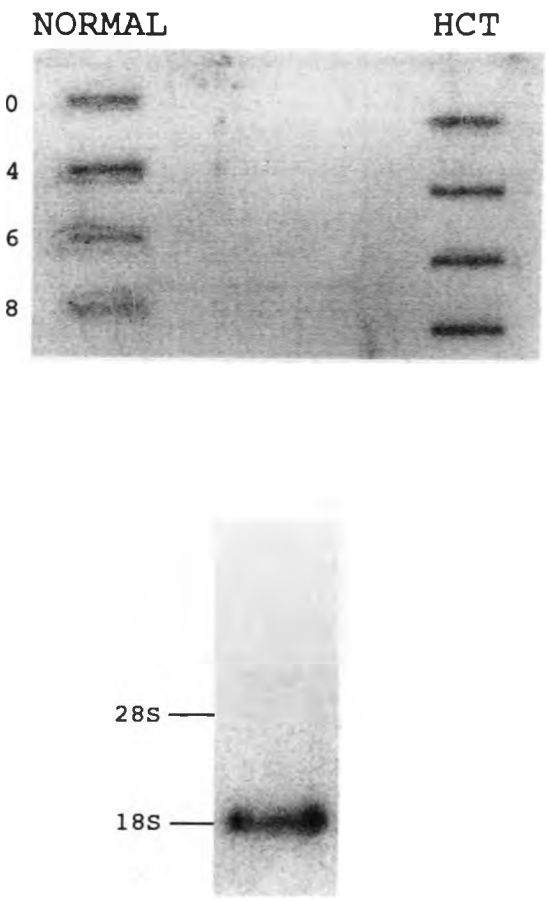

Figure 20: Autoradiographs of slot blots of RNA from macrophages cultured in the presence of iron for various time intervals of 1 normal and 1 haemochromatotic subject (HCT) probed for ß-actin mRNA. The lower panel demonstrates a northern blot showing probe specificity.

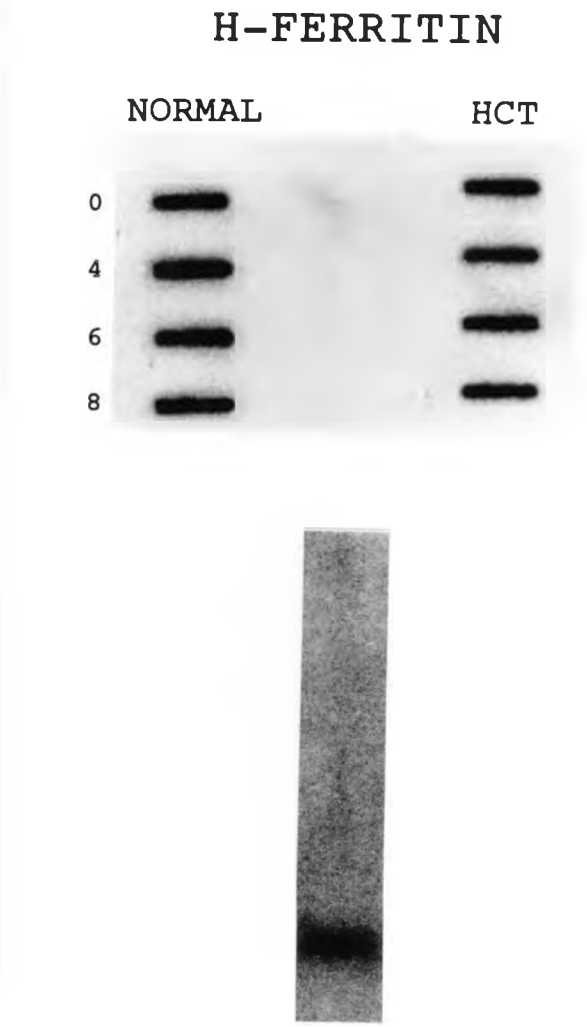

Figure 21: Autoradiographs of slot blots of RNA from macrophages cultured in the presence of iron for various time intervals of 1 normal and 1 haemochromatotic subject (HCT) probed for H-ferritin mRNA. The lower panel demonstrates a northern blot confirming probe specificity.

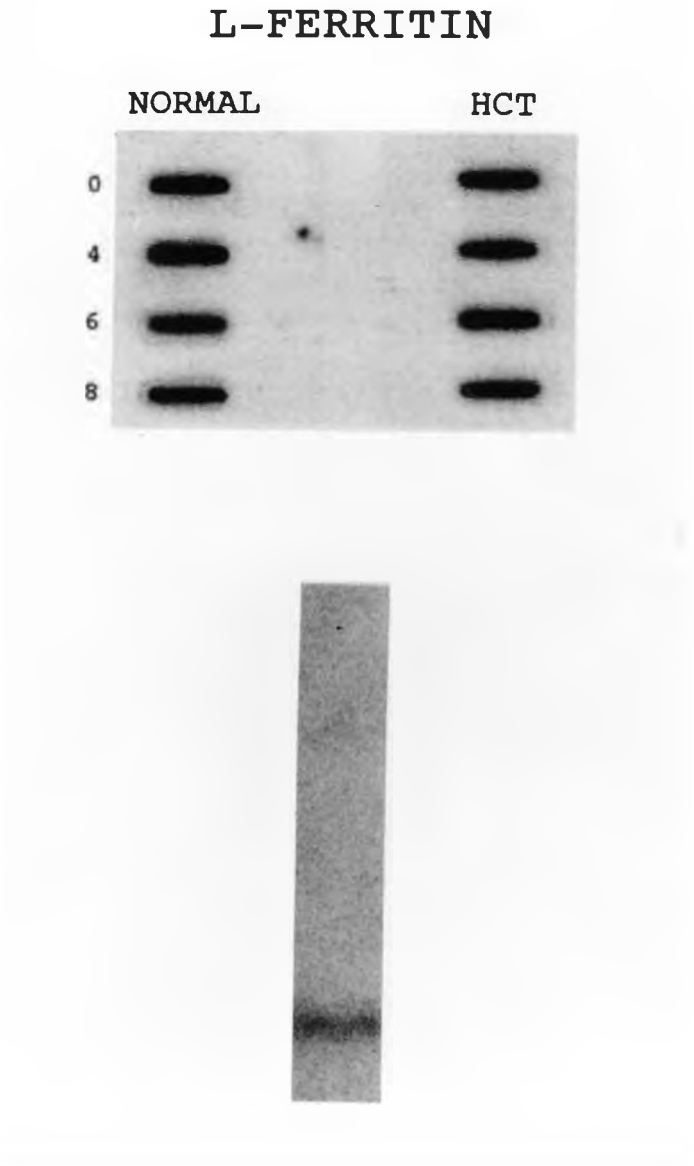

Figure 22: Autoradiographs of slot blots of RNA from macrophages cultured in the presence of iron for various time intervals of 1 normal and 1 haemochromatotic subject (HCT) probed for L-ferritin mRNA. The lower panel demonstrates a northern blot showing probe specificity.

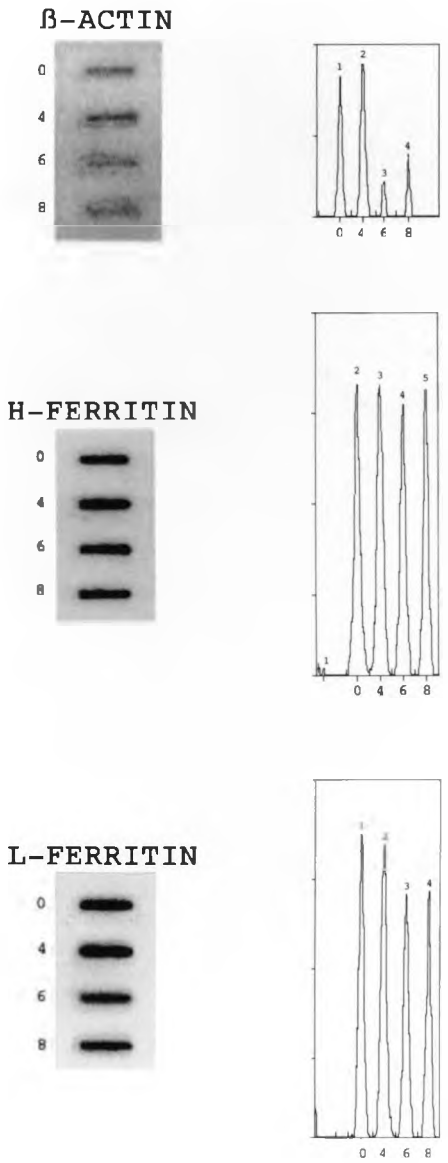

Figure 23: Slot blot analysis by densitometric scanning of normal macrophage RNA probed with ß-actin, H-ferritin and L-ferritin.

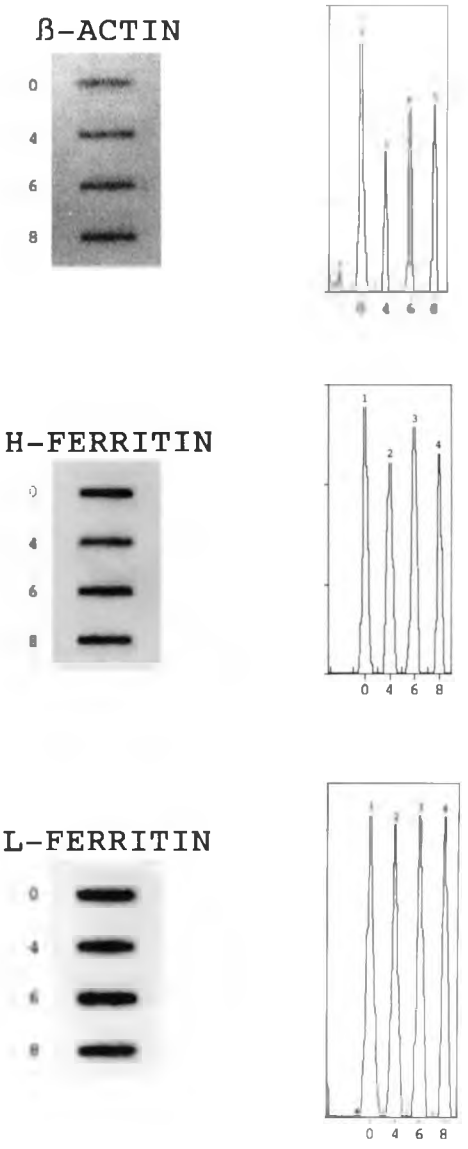

Figure 24: Slot blot analysis by densitometric scanning of HCT macrophage RNA probed with ß-actin, H-ferritin and L-ferritin.

The area under each peak was calculated by computer analysis and the values for the H-ferritin to ß-actin mRNA ratios and the L-ferritin to ß-actin ratios are shown in Tables IV and V, respectively. (The values for H- and L-ferritin mRNAs were expressed relative to ß-actin mRNA to account for any variation in the amount of RNA added to each slot). The calculated ratios of ferritin mRNA to ß-actin mRNA signals (values at t=0 normalized to 1) were plotted against time of incubation with iron and the results are depicted in figures 25 and 26. Both normal and haemochromatotic cells show an increase in H and L mRNA to ß-actin but the patterns are different. In normal macrophages (figure 25) the ratio of H-ferritin mRNA to ß-actin mRNA peaked after 6 hours of incubation with iron. In haemochromatotic macrophages the peak was at 4 hours and was about half that seen with normal macrophages. Thereafter the levels returned to baseline values for both study groups. This was confirmed when the incubation time was extended to 24 hours (not shown on graph). A greater increase in L-ferritin mRNA relative to ß-actin mRNA at 6 hours was also observed in normal macrophages compared with haemochromatotic macrophages (figure 26). No changes in the ratios of L-ferritin to H-ferritin mRNA were noted in cells from either normal subjects or patients with haemochromatosis.

TABLE IV : Ratios of H-ferritin mRNA to ß-actin mRNA for 1 normal and 1 haemochromatotic (HCT) subject.

Time (hours)	Normal	HCT
0	2.88	1.16
4	2.24	2.90
6	12.84	2.11
8	7.17	1.48

TABLE V : Ratios of L-ferritin mRNA to ß-actin mRNA for 1 normal and 1 haemochromatotic (HCT) subject.

Time (hours)	Normal	HCT
0	2.90	1.96
4	2.27	4.37
6	11.99	3.08
8	6.09	2.65

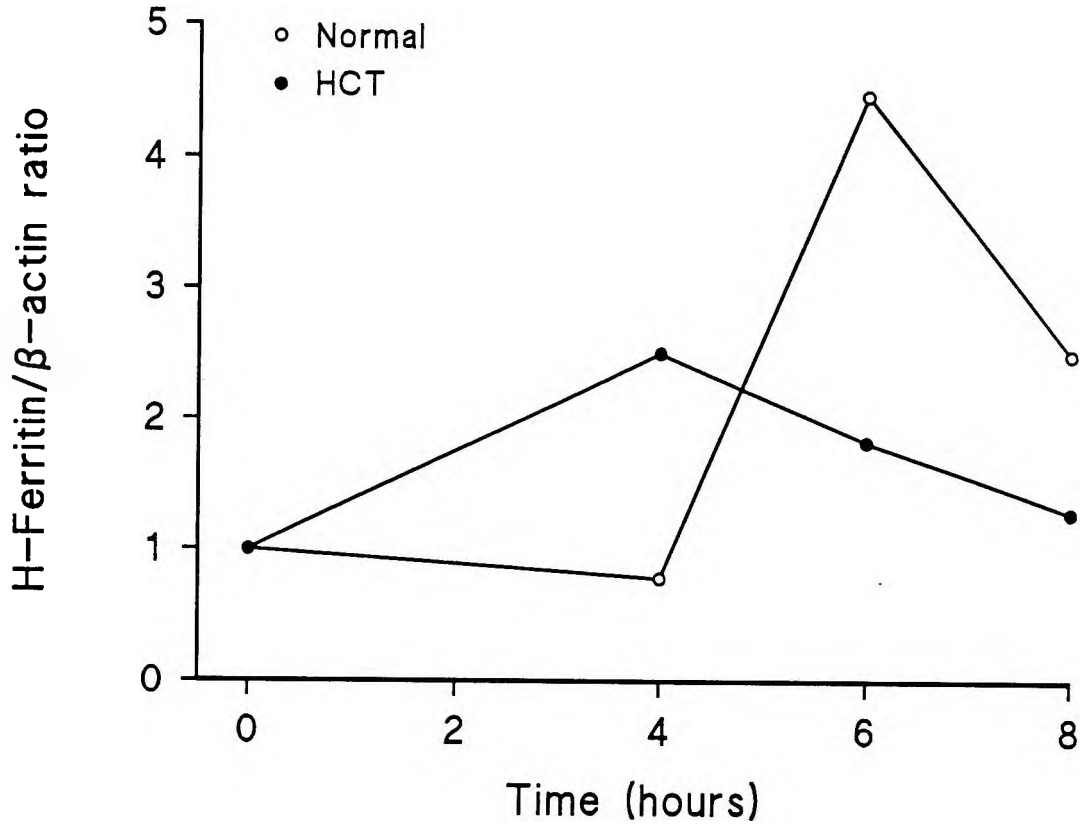

Figure 25: The ratios of H-ferritin mRNA to ß-actin mRNA with respect to time of incubation of cultured macrophages from 1 normal (o) and 1 haemochromatotic subject (●) with iron. Quantitative analyses of all densitometric data obtained from Table IV.
(Values have been corrected such that the densities at time 0=1)

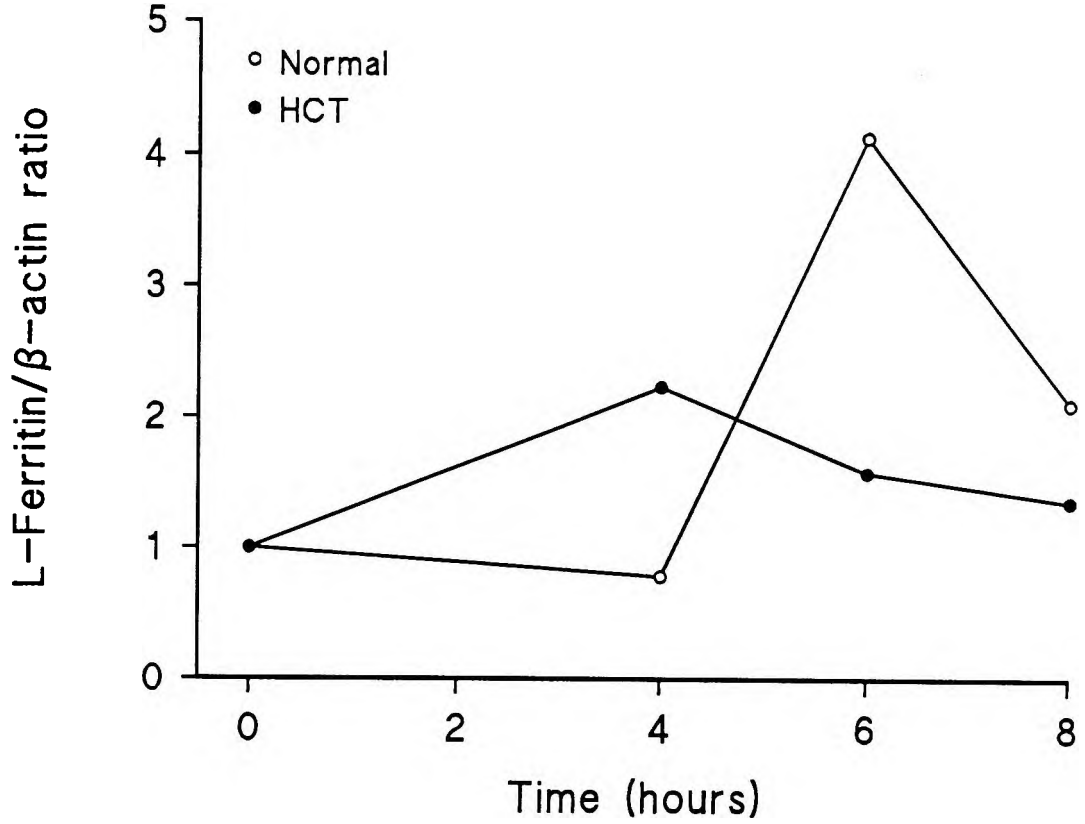

Figure 26: The ratios of L-ferritin mRNA to ß-actin mRNA with respect to time of incubation of cultured macrophages from 1 normal (o) and 1 haemochromatotic subject (●) with iron. Quantitative analyses of all densitometric data obtained from Table V.
(Values have been corrected such that the densities at time 0=1)

5.4 DISCUSSION

RE cells in hereditary haemochromatosis appear to be spared in terms of iron accumulation (Valberg et al, 1975; Brink et al, 1976; McClaren, 1989). It has been proposed that these cells are unable to synthesize ferritin adequately resulting in a failure to retain iron normally causing enhanced absorption and iron deposition in parenchymal cells. Because of this, the present in vitro study aimed to investigate whether macrophage ferritin mRNA levels in response to iron administration differed between normal subjects and haemochromatotic patients.

Firstly, the level of both H- and L-ferritin mRNA is seen to rise relative to ß-actin for both normal and haemochromatotic cells. The pattern of change for the two types of cells appear to be different in that the haemochromatotic cells show an approximate 2 fold increase by 4 hours, followed by a decrease to approximate the initial level at 8 hours, while the normal cells do not show an increase for 4 hours, then a large (4 to 5 fold) increase by 6 hours which falls by 8 hours. The study by Testa and co-workers (1989) demonstrated a marked increase in H- and L-ferritin mRNA in monocytes cultured for 8 days compared with fresh monocytes. In the present study, only 8 day macrophages were studied and therefore no comparison between ferritin mRNAs in normal and haemochromatotic cells can be made on the changes during the in vitro maturation process. The same authors also observed a slight increase in ferritin mRNA in response to iron but

far greater increases in the total ferritin content, implying that a translational mechanism may be involved. The iron induced increase in ferritin mRNAs noted in the current study may have occurred as a result of an enhanced transcription and/or increased stability of the transcripts. It is possible that the cells from both study groups were relatively deprived of iron after 8 days in culture and transcriptional activation resulted in the synthesis of more ferritin subunit mRNAs for iron storage for intracellular use. It should however be noted that a possible role of translational activation was not considered here, since the total ferritin content was not determined.

The greater increase in H- and L-ferritin mRNA levels observed in normal macrophages compared with cells from patients with haemochromatosis needs to be explained. The addition of iron salts to monocyte-macrophage cultures (from healthy subjects) has been shown to moderately enhance the levels of ferritin mRNA subunits (Testa et al, 1989). This mechanism whereby iron induces ferritin expression appears to be less effective in haemochromatotic cells. It is possible that haemochromatotic macrophages fail to respond normally to iron given in this form. However, previous workers have demonstrated normal ferritin protein synthesis in macrophages in response to iron presented as ferric ammonium citrate (Bassett et al, 1982; Testa et al, 1989). In the previous study on transferrin-iron-macrophage interactions (section 2,

chapter 3), the uptake of iron from transferrin by haemochromatotic macrophages was lower than by normal macrophages. This may be explained by an increased release of iron by haemochromatotic macrophages and hence lower intracellular iron levels. If the same mechanism applies when iron is presented as ferric ammonium citrate, the difference in ferritin mRNA levels could also be explained on the basis of lower intracellular iron causing less ferritin gene expression in haemochromatotic macrophages. The abnormal behaviour of the RE cell should be paralleled to that of the intestinal mucosal cell since the latter is also relatively spared of iron (Brink et al. 1976). Insofar as the gut is concerned, McClaren and co-workers (1991) showed that an increased mucosal iron transfer rate was the major determinant of increased iron absorption in hereditary haemochromatosis. In another study performed on duodenal mucosal cells of patients with haemochromatosis, reduced levels of ferritin subunit mRNAs and a lack of transferrin receptor down-regulation was postulated to occur secondary to lower mucosal iron content caused by accelerated release of cellular iron (Pietrangelo et al. 1992). It therefore appears that the gut and RE cells may share a common defect of impaired iron release in HLA-linked haemochromatosis.

The present study is only concerned with measuring the early gene products (ferritin subunit mRNAs). In order to comment on ferritin synthesis as a whole, the amount of the final protein product, ferritin, the distribution of

free to polysome-bound mRNA and the rate of ferritin mRNA turnover would need to have been measured, but was beyond the scope of this study. Furthermore, a larger study group would have to be investigated to confirm the current findings. The refinement and application of molecular biological techniques to this study has however been a most valuable learning experience.

6. CONCLUSION

HLA-linked haemochromatosis is associated with an altered distribution of storage iron. The RE cells and intestinal mucosal cells release more iron than is needed. It is known that RE cells derive most of their iron from senescent red blood cells and that iron delivered by transferrin represents a small fraction of RE turnover. In the present study, the uptake of transferrin and transferrin iron was determined in cultured macrophages from normal subjects and patients with haemochromatosis. An increased transferrin uptake was noted in macrophages from patients with haemochromatosis compared to normal macrophages. However, the uptake of iron from transferrin was notably less in haemochromatotic macrophages. This could be explained by an inability of RE cells to store iron as ferritin in haemochromatosis. It is also possible that the observed response may relate to an accelerated release of iron to the extracellular environment leading to lower intracellular iron levels.

It is known that ferritin plays a central role in the storage of iron and that its synthesis is regulated by

iron. In vitro studies were performed to evaluate the effect of iron on the levels of ferritin subunit mRNAs in normal and haemochromatotic cultured macrophages. The expression of both heavy and light ferritin chains at RNA level was greater in normal macrophages compared to haemochromatotic macrophages. The findings suggest a primary defect in ferritin mRNA expression in response to iron in RE cells from patients with haemochromatosis. Alternatively, the reduced levels of ferritin mRNA transcripts are presumably secondary to decreased cellular iron content attributable to enhanced cellular iron release. The present study did not address the effect of iron on post-transcriptional processes, the rate of ferritin mRNA turnover and the amount of the final gene product, ferritin. This may provide further insight into the theory that ferritin synthesis is impaired in mononuclear phagocytes in hereditary haemochromatosis.

On the basis of the present study one might speculate, therefore, that the paucity of iron observed in the RE cells in haemochromatosis may relate to increased iron release from the cells. The cells may then respond to an apparent iron deficient state by lowering ferritin gene expression as ferritin mRNA. The hypothesis may not be mutually exclusive if it is assumed that the RE cells and intestinal mucosal cells behave in the same way. A common defect in the cellular and molecular mechanisms in either iron storage or the release of increased amounts of iron to the circulation may be postulated to be central to the disordered iron metabolism of HLA-linked haemochromatosis.

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