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RETICULOENDOTHELIAL FERRITIN MESSENGER RNA IN INFLAMMATORY STATES

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree Master of Science in Medicine.

Johannesburg, 1989.

DECLARATION

This dissertation is my own unaided work, and no part of it has previously been presented at this or any other University. The information used in this dissertation was obtained while I was employed by the University of the Witwatersrand, the Johannesburg Hospital and the Medical Research Council of South Africa, in the MRC Iron and Red Cell Metabolism Research Unit.



S.E. Lapinsky

ABSTRACT

Ferritin is an iron storage protein, made up of heavy (H) and light (L) subunits. Ferritin synthesis is regulated at a post-transcriptional level by iron, which induces a redistribution of ferritin mRNA from a free cytoplasmic pool to polyribosomes. Inflammatory states influence iron metabolism, causing a decrease in serum iron levels associated with an increase in reticuloendothelial ferritin synthesis and iron storage. It has not been established whether this stimulation of ferritin synthesis occurs as a primary event or secondary to a rise in intracellular iron levels, nor whether this is regulated at a transcriptional or translational level.

This study investigates reticuloendothelial ferritin mRNA changes in inflammatory states. In the first study, RNA was extracted from blood monocytes of patients with acute inflammation (lobar pneumonia) and chronic inflammation (pulmonary tuberculosis) as well as from normal controls. This RNA was probed for ferritin subunit mRNA with β-actin acting as a constitutively expressed control. No increase in steady state levels of either H- or L-ferritin subunit mRNA was found. A second study investigated changes in ferritin mRNA in hepatic and splenic tissue of rats with inflammation induced by turpentine injection. A significant decrease in hepatic L-ferritin mRNA and a slight, but not statisically significant, decrease in splenic L-ferritin mRNA was noted. A trend towards an increased H- to L-ferritin mRNA ratio was found in both hepatic and splenic tissue during the 12 hours after injection. Although no change in the control β-actin mRNA occurred in reticuloendothelial tissue, a

marked increase was found in hepatic tissue, suggesting transcriptional stimulation of actin synthesis in inflammation. Subsequent investigation of the redistribution of the cytoplasmic pool of ferritin mRNA in rat splenic tissue did not yield conclusive data.

These studies suggest that the increase in ferritin synthesis during acute inflammation is post-transcriptionally regulated, with differential regulation occurring for the H- and L-ferritin subunits. The mechanism of ferritin synthesis in inflammation therefore differs from the transcriptional mode of synthesis of other acute phase proteins.

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1. INTRODUCTION

1.1 THE RETICULOENDOTHELIAL SYSTEM

1.1.1 Introduction

The reticuloendothelial system is a mononuclear phagocyte system located primarily in the reticular connective tissue framework of the spleen, liver and lymphoid tissue but with representative cells in most organs. It is made up of a number of histologically heterogeneous cells, namely the circulating monocytes which originate in the bone marrow and which differentiate into tissue macrophages. These include the epidermal Langerhans cells, alveolar macrophages, pleural and peritoneal macrophages, and microglial cells of the central nervous system. The Kupffer cells of the liver constitute a large proportion of the cells of the reticuloendothelial system and other intravascular cells include the lining macrophages of the sinuses of spleen and lymph nodes (Nathan, 1987). Monocytes have a circulating half life of about 3 days and while the lifespan of tissue macrophages is not known, it is probably months rather than days (Auger, 1989).

This macrophage system has a number of important functions, namely phagocytosis, secretion of biologically active products and a central role in immune responses. The phagocytic function includes uptake of microorganisms and these cells play an important role in defence against a number of pathogens (Cline et al, 1978). Macrophages of the liver and spleen are responsible for removal of senescent and damaged circulating blood cells, as well as clearance of drugs, endotoxin and fibrin (Saba, 1970). In addition to removal of red blood cells, the reticuloendothelial system plays a central role in the storage and recirculation of iron (Lynch et al, 1974).

Macrophages secrete more than 100 substances involved in the acute phase response, regulation of haemopoiesis and activation of the immune and coagulation systems (Auger, 1989). These secretory products include enzymes such as proteases, lysozyme and acid hydrolases; polypeptide hormones including interleukin-1, tumour necrosis factor-α , interferon-α , granulocyte-macrophage colony stimulating factor, β -endorphin, and ACTH; complement components C1, C4, C2, C3, C5, and factors B, D, properdin and C3b inactivator; enzyme inhibitors; binding proteins such as transferrin, transcobalamin II and apolipoprotein E; bioactive lipids such as PGE_2, thromboxane, leukotrienes and platelet activating factor, and reactive oxygen species (Nathan et al, 1980; Nathan, 1987).

Macophages are essential for most immunological reactions. They initiate the inflammatory response by presenting antigen to lymphocytes (Unanue and Allen, 1987) and their secretory products modulate lymphocyte function. These cells are responsible for the removal of immune complexes and they are responsible for the formation of granulomata, congregating in most subacute and chronic reactions (Nathan, 1987). Activation of monocytes by endotoxin, immune complexes, complement and γ -interferon results in enhanced function and secretory activity (Auger, 1989).

1.1.2 Iron metabolism in reticuloendothelial cells

1.1.2.1 Phagocytosis of haemoglobin iron

Haemoglobin iron from senescent red blood cells is recirculated to the developing erythron via the reticuloendothelial system. Reticuloendothelial cells obtain iron almost solely from phagocytosis of red cells and virtually no iron enters these cells from the plasma

transferrin pool. This transfer of iron from the erythron to the reticuloendothelial system occurs predominantly at the time of red cell death, but also with extrusion of the red cell nucleus and expulsion of storage iron granules (Bothwell et al, 1979).

The phagocytosis of senescent red cells is a function of the cordal macrophages of the splenic red pulp, although this has only been demonstrated experimentally using infusions of damaged red cells, and has not been demonstrated in the normal situation (Deiss, 1983). The physical stress of passage of erythrocytes through small pores in splenic cords may result in destruction of aged cells with decreased membrane deformability (Chen and Weiss, 1972). In certain situations, phagocytosis of red cells has been demonstrated by reticuloendothelial cells in the liver and bone marrow (Hugh Jones, 1961; Frank et al, 1977). Sites other than the spleen may remove senescent erythrocytes in haematologically normal subjects after splenectomy (Gevirtz et al, 1962). Free haemoglobin, haemoglobin-haptoglobin complexes and haem-haemopexin complexes are removed by hepatocytes or renal cells and not by reticuloendothelial cells (Hershko et al, 1972).

1.1.2.3 Intracellular events

Iron is released from haemoglobin in the reticuloendothelial cell by the action of microsomal haem oxygenase (Tenhunen, 1972). The greatest activity of this enzyme is found in splenic macrophages. After destruction of the phagocytosed red blood cell, globin is split from the haemoglobin molecule and the enzyme degrades the resulting haemin. Iron is released, with the formation of biliverdin and carbon monoxide.

Once iron is freed from the haemoglobin molecule, it may return to the plasma or become incorporated in iron stores as ferritin or haemosiderin. Intermediate storage pools may also exist, but have not been well characterised. The majority of this iron (85%) is released to transferrin (Noyes et al, 1960), but mixing with the storage iron pool does occur over a period of time (Green et al, 1968). Interchange with the storage pool can vary depending on the iron status of the individual (Lynch et al, 1974).

1.1.2.4 Iron release

The release of iron appears to occur in a biphasic manner. An initial early release phase with a half time of release of 34 minutes is followed by the release of the remaining iron, diverted through a late release pool. Approximately one third of the phagocytosed iron passes through this latter pool and leaves the cell with a half time of 7 days (Fillet et al, 1974). Little is known about the nature of the intermediate iron pools or the actual mechanism of release (Deiss, 1983), but a number of factors are known to influence this process.

The availability of transferrin is necessary for release of iron by the reticuloendothelial cell (Solvell, 1960) and it has been demonstrated that venesection enhances early release while hypertransfusion diminishes iron release (Lipschitz et al, 1971b; Fillet et al, 1974). Caeruloplasmin acts as a ferroxidase and is necessary for the conversion of iron from the ferrous to the ferric form, the oxidation state bound by transferrin (Frieden and Osaki, 1974). Ascorbic acid has also been demonstrated to be essential for release of storage iron (Lipschitz et al, 1971a). Other determinants of the quantity of iron release include a circadian variation (Lynch

et al, 1973) and control in some way by marrow requirements. Hormonal mechanisms probably play no role in control of plasma iron (Lynch et al, 1974).

1.1.2.5 Regulation of iron release

Plasma iron levels appear to be regulated passively. Transferrin bound iron moves into normoblasts and hepatocytes at a rate dependent on the plasma iron concentration (Skarberg et al, 1978). The mechanisms of regulation of the flow of iron from the reticuloendothelial cell to plasma are not clear. In iron deficiency, all haemoglobin iron is released and up to 100 mg per day of the stored iron may be mobilized (Noyes et al, 1960). A direct feedback effect of plasma iron on reticuloendothelial iron release has been reported but transferrin saturation was produced unphysiologically in these studies (Lipschitz et al, 1971b; Bergamaschi et al, 1986). Other studies have reported no feedback control by plasma iron levels (Fillet et al, 1974). With more physiological saturation of transferrin, by ingestion of carbonyl iron, no effect on release of iron by splenic tissue has been found. Iron release from hepatocytes, however, is inhibited by this saturated transferrin possibly due to the higher density of transferrin receptors on these cells (Siegenberg et al, submitted). Transfer of iron from splenic macrophages to hepatocytes in the form of ferritin or low molecular weight iron complexes may occur in significant amounts in the presence of saturated transferrin.

1.2 FERRITIN

1.2.1 Introduction

Ferritin is an iron storage protein found in animal, plant (Crichton et al, 1978), fungal (David and Easterbrook, 1971) and bacterial cells (Stiefel and Watt, 1979). The protein was first crystallized in 1937 by Laufberger, from horse spleen and Granick subsequently isolated ferritin from several species. He confirmed its iron storage role, also suggesting an additional role in iron absorption (Drysdale, 1977). Ferritin is abundant in liver, spleen and bone marrow, where it plays an important role in recycling and storage of iron. It also has a protective function in sequestering toxic "free" iron, which has the ability to denature proteins and to produce free radicals. The vital role of this protein is demonstrated by the fact that apoferritin synthesis is not impaired even in the presence of severe protein malnutrition (Achmed and Ramsay, 1974). In addition to its importance in understanding iron metabolism, ferritin has provided a model for researchers studying multi-subunit proteins, intramolecular channels, and translational regulation of protein synthesis.

1.2.2 Structure

Ferritin is a multi-subunit protein approximately 12 nm in diameter, made up of a spherical protein coat with molecular weight 450 000 (Harrison et al, 1977), enclosing a core of hydrous ferric oxide - Fe(III)O·OH (Aisen, 1977). Apoferritin has been isolated from ferritin preparations or may be prepared by reduction of the ferric core followed by chelation (Crichton et al, 1973; Chasteen and Theil, 1982). The apoprotein coat has been shown by X-ray crystallographic

studies to be made up of 24 subunits (Hoare and Harrison, 1975) with four-, three-, and two-fold axes of symmetry. Each ferritin subunit consists of four long alpha helices, named A, B, C and D, with a short terminal helix E (Harrison et al, 1987). Although these subunits are heterogeneous (see below), structural homology of the ferritin protein as a whole is maintained as determined by X-ray diffraction patterns (Rice et al, 1985). Certain sequences of amino acids appear to be highly conserved from amphibians to mammals and these confer specific properties on the protein. Intersubunit salt bridges and hydrogen bonds account for the thermostability of ferritin. Six hydrophobic channels, lined with leucine residues, are situated at the four-fold E-helix ends, and eight hydrophilic channels made up of the three-fold N-terminal ends occur (Banyard et al, 1978; Ford et al, 1984). The latter channels contain highly conserved amino acid residues, Asp-127 and Glu-130, and are believed to be the route of transport of iron in and out of the ferritin molecule (Clegg et al, 1980). A number of modifications of subunits such as glycosylation and covalent crosslinks may occur, probably related to the physiological state of the cell (Mertz and Theil, 1983).

The central iron core consists of hydrous ferric oxide, with varible amounts of phosphate. Each molecule of ferritin can accomodate up to 4 500 iron atoms, but the average iron content is about 2 500 atoms (Niitsu and Listowsky, 1973). A number of other monoatomic metal ions are bound by apoferritin, including Cd(II), Zn(II), Mn(II), Tb(III), UO(III) and VO(IV) (Theil 1987). Iron is not randomly distributed in the core, but is located in microcrystalline particles on the inner surface of the apoprotein coat (Harrison et

al, 1987). In the presence of low iron concentrations, Fe(II) probably binds to the three-fold channels where it is oxidized to Fe(III), then entering the cavity. If excess iron is present, Fe^{2+} ions may pour into the cavity and oxidize directly on the iron oxide crystals (Harrison et al, 1987). Harrison has postulated that oxygen and inorganic phosphate may enter the core via the 4-fold channels.

1.2.3 Isoferritins

Heterogeneity of ferritin was first demonstrated electrophoretically by starch gel electrophoresis in 1963 (Kopp et al, 1963). However this was not true heterogeneity, being due to molecular aggregates forming dimers, trimers and tetramers, as evidenced by sedimentation studies (Harrison and Gregory, 1965) and electron microscopy (Williams and Harrison, 1968). True isoferritins were demonstrated electrophoretically and serologically by Richter (1964), comparing HeLa ferritin with other ferritins. Variation in ferritins purified from normal human tissues was subsequently demonstrated (Alfrey et al, 1967) and was shown to be due not to differences in iron content but to differences in subunit type (Drysdale, 1970). Isoelectric focusing demonstrated greater complexity even in electrophoretically "pure" ferritin (Drysdale 1970), and these isoferritins did not redistribute when refocused (Alpert et al, 1973). Heterogeneity can also be resolved by ion-exchange chromatography (Drysdale, 1974) and by gel filtration (Drysdale, 1977). Amino acid analysis and tryptic peptide mapping (Lee and Richter, 1971; Crichton et al, 1973) also demonstrated differences between ferritins from many tissues, both normal and malignant.

A structural basis for this heterogeneity was proposed by Drysdale (1970), who suggested that isoferritins are hybrid structures made up of a few common subunit types. He subsequently proposed a 2-subunit hypothesis based on acid urea gel electrophoresis (Adelman et al, 1975) and this was later confirmed and expanded upon (Arosio et al, 1978). The 2 subunits were designated "H" (heart, heavy) and "L" (liver, light) with molecular weights of approximately 21000 and 19000 respectively. Isoferritins are made up of various ratios of H and L subunits, resulting in differences in size, amino acid content, charge and immunoreactivity. Acidic isoferritins are made up of predominantly H-subunits, and basic isoferritins have a predominance of L-subunits (Powell et al, 1975). Isoferritin profiles show an organ specific variation, with acidic isoferritins predominating in the heart, kidneys and monocytes, and basic isoferritins in the liver and spleen (Powell et al, 1975). Liver ferritin contains approximately 70 to 90% L-subunits while heart ferritin has 40 to 70% H-subunits (Hazard et al, 1977; Arosio et al, 1976; Drysdale, 1977). The pI range for isoferritins from human tissues is from 5,06 to 6,04 (McKeering et al, 1976). Immunological differences between isoferritins have been shown to parallel H- and L-subunit content (Hazard et al, 1977). The H to L subunit ratio varies between tissues but may also be altered during cellular differentiation or in pathological states such as neoplasia (Chou et al, 1986; Drysdale et al, 1977). The functional significance of these changes is not clear but there is evidence that the L-subunit rich isoferritins have a higher iron storage capacity (Bomford et al, 1978).

Recent cloning of the cDNA for ferritin subunits has allowed a new approach to the investigation of isoferritins (Constanzo et al, 1984; Dorner et al, 1985). Boyd and co-workers (1985) cloned full length human cDNAs for both subunits from a human liver library. The H-subunit cDNA was identified by hybrid select translation and immunoprecipitation, and the L-subunit cDNA by its similarity to the horse spleen ferritin L-chain sequence. The calculated molecular mass of the H-subunit is 21 099 dalton (182 amino acids) and of the L-subunit is 19 766 dalton (175 amino acids), which corresponds well with those determined by SDS gel electrophoresis. Sequencing of the cDNA has demonstrated a 55% homology between the two chains.

Although the 2 subunit hypothesis is generally accepted, the complexity of the ferritin subunit structure may be greater than can be accounted for by this model (Theil, 1987). Three mRNAs have been identified encoding 3 subunits in HeLa cells (Watanabe and Drysdale, 1983) and in bullfrog red cells (Dickey et al, 1987), and the pig liver L-subunit has been resolved into 3 fractions by reverse phase high performance liquid chromatography (Collawn and Fish, 1984). In addition, 8 sequences similar to the L-cDNA and 12 sequences similar to the H-subunit gene have been identified in the human genome (Jain et al, 1985). These may represent pseudogenes or may be genes for additional ferritin subunits.

1.2.4 Functions

Ferritin acts as an iron storage protein, maintaining ferric iron in a soluble form. In the presence of oxygen, iron exists in a ferric state, which is insoluble in concentrations greater than 10^{-18} molar (Bullen et al, 1978). Theil (1987) has differentiated ferritin

function into storage of iron for use by other cells (specialized cell ferritin), for intracellular use (housekeeping ferritin), and for detoxification (housekeeping stress ferritin). Whether the ferritins subserving these various functions are structurally distinct has not been established. Specialized cell ferritin has a number of different roles in the provision of iron for other cells. The ferritin in red cells of embryos provides a pool of iron available for rapid consumption (Dickey et al, 1987) while long term storage of iron occurs in hepatocytes. Macrophages recycle haemoglobin iron, making it available for the erythron. Approximately 30 mg of iron is generated daily by recycling from senescent red cells and from cellular wastage during erythropoiesis, and this is returned to the plasma (Cook et al, 1970). In addition, these cells also contain ferritin serving a housekeeping function, and ferritin playing a role in the stress response to inflammation (Mertz and Theil, 1983). Housekeeping ferritin provides iron for cytochromes, ribonucleotide reductase, haemoglobin and myoglobin. Although structurally distinct ferritins have not been demonstrated, it has been shown that the physiological state of the cell may modify ferritin, allowing a change in the steady state content of iron (Mertz and Theil, 1983).

The uptake of iron by ferritin is a complex process, whereby ferrous iron is oxidized to ferric iron, a reaction catalysed by the ferritin protein (Bryce and Crichton, 1973). This appears to be function of the carboxyl groups in the region of the B-helix near the two-fold axis of symmetry. Initially, nucleation is a slow process, affected by modification of the protein (Chasteen and Theil, 1982) and the presence of other metals such as zinc. This is followed by a

period of rapid growth of the iron core (Macara et al, 1972), where iron uptake occurs as an inorganic reaction dependent on the availability of iron. After approximately 1000 atoms of iron have entered the ferritin core, the reaction slows (Aisen, 1977). The release of iron from ferritin is dependent on the rate of reduction of ferritin iron, and is altered by pH, the age of the protein coat (Watt et al, 1985) and crosslinks between subunits (Mertz and Theil, 1983).

Ferritin also plays an important role in protecting the organism from toxic free iron. Iron catalyses the production of active oxygen species (McCord and Day, 1978) which mediate tissue damage by lipid peroxidation of membranes (Halliwell and Gutteridge, 1984). Active hydroxyl radicals (OH·) are produced by the reduction of H_2O_2 by divalent metal ions :

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

As the concentration of Fe^{2+} becomes limiting, reduction of Fe^{3+} by superoxide radicals (O_2^-), found in all aerobic cells, is required:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

The overall reaction is therefore:

$$H_2O_2 + O_2^- \xrightarrow[Fe^{2+}]{} O_2 + OH^- + OH\cdot \quad \text{(Haber-Weiss reaction)}$$

Although storage of iron in ferritin is believed to sequestrate the iron, protecting the cell from these reactions, it has been suggested that ferritin may provide a source of iron for catalysing production of hydroxyl radicals (Thomas et al, 1985; Koster and Slee, 1986). This would probably only occur in the diseased cell or if superoxide levels were abnormally high.

A number of other roles for ferritin have been suggested. Ferritin contains 4 to 10 atoms of copper and zinc per molecule, and

may play a role in storage of these trace elements (Price and Joshi, 1982). Oxidation of ferrous iron in the production of the iron core releases up to 4 500 electrons and 11 250 H^+. In bacteria such as Thiobacillus ferrooxidans this is coupled to energy producing reactions, but an energy generating function for ferritin has not been defined. Ferritin is found in low concentrations in the serum, and the source and functions of this serum ferritin have not been clearly established (Worwood 1986). Monocyte derived acidic isoferritins have been shown to regulate myelopoiesis in vitro (Broxmeyer et al, 1982 & 1986), and serum ferritin may play a role in suppression of T-lymphocyte function (Matzner et al, 1979 & 1985).

1.2.5 Synthesis

1.2.5.1 Introduction

The regulation of synthesis of most proteins occurs by means of changes in mRNA concentration, mediated by a variation in the rate of transcription or in stability of the mRNA. Ferritin synthesis is known to be induced by iron (Fineberg and Greenberg, 1955). This stimulatory effect involves a post-transcriptional mechanism as demonstrated by the observation that Actinomycin D, an inhibitor of RNA synthesis, and cordycepin, an inhibitor of mRNA processing, do not prevent iron-induced ferritin synthesis (Drysdale and Munro, 1965; Zahringer et al, 1976a). Two post-transcriptional steps have been suggested. Zahringer and co-workers (1976a) proposed that ferritin mRNA occurs in the cytoplasm in a repressed form and this is recruited by iron for translation. Shull and Theil (1982) observed that this is combined with highly efficient competitive translation of the derepressed ferritin mRNA.

Post-transcriptional regulation of protein synthesis is unusual but has been shown to occur in a number of other proteins. These include the production of pro-insulin in rats (Itoh and Okamoto, 1980), synthesis of mouse apolipoprotein E (Reue et al, 1984) and rat liver sterol carrier protein (McGuire et al, 1984). The proteins produced during heat shock in Drosophila have been shown to be translationally regulated (Lindquist, 1981) as have proteins produced during embryogenesis (Frusculoni et al, 1983). The activation of pre-existing untranslated mRNA probably provides a quicker response than gene activation, allowing rapid sequestration of excess iron. Shull and Theil (1983) have suggested that entry of iron into the nucleus to activate transcription could damage DNA and this has been demonstrated experimentally (Loeb et al, 1988). A post-transcriptional mechanism of regulation prevents repeated interaction of genomic DNA with iron.

The intracellular site of protein synthesis depends on whether the protein is retained for intracellular use, or is exported to the plasma. Proteins synthesized for export are made predominantly on ribosomes bound to the membrane of the endoplasmic reticulum, while proteins for intracellular use are synthesized by ribosomes free in the cytosol. Hicks and colleagues (1969) demonstrated that ferritin is synthesized preferentially on free ribosomes and this was confirmed by Lee and Richter (1977) who found that only 20% of ferritin was made by membrane-bound ribosomes. Induction of ferritin synthesis by iron causes an increase only in free ribosome-derived ferritin (Konijn et al, 1973).

1.2.5.2 Post-transcriptional regulation

Studies of ferritin mRNA regulation have taken advantage of the fact that iron induces ferritin synthesis in specialized storage cells and it is in these cells that post-transcriptional regulation has been observed. Zahringer and co-workers (1975) first demonstrated that the amount of functional ferritin mRNA, assessed by means of cell free translation, did not increase on stimulation by iron. They also demonstrated a substantial cytoplasmic pool of ferritin mRNA. Although 15% of total rat liver poly(A)-RNA was found in the post-microsomal supernatant, this was highly enriched with ferritin mRNA, containing 40-50% of the total cytoplasmic ferritin message (Zahringer et al, 1976b). Recruitment of this ferritin mRNA to polysomes was also observed after exposure to iron (Zahringer et al, 1976a). Shull and Theil (1982) confirmed the translational control of ferritin synthesis, demonstrating that iron acts by increasing the availability of functional ferritin mRNA for translation. Animal studies (Aziz and Munro, 1986) and acute in vitro studies using cultured rat hepatoma cells (Rogers and Munro, 1987) have shown that the increase in ferritin production induced by iron is due to redistribution of ferritin mRNA from an inactive cytoplasmic pool to translationally active polyribosomes. Following stimulation by iron, the free ferritin mRNA pool decreased from 65% of the total cytoplasmic ferritin mRNA to 15%, with a corresponding increase in polyribosome-bound ferritin mRNA. No increase in total cellular ferritin mRNA was noted. These latter two studies used subunit specific cDNA probes and found similar changes for both the H and L ferritin subunits. Rogers and Munro (1987) also demonstrated that it was the level of chelatable iron within the cell that regulated

ferritin mRNA distribution. A recent report by White and Munro (1988), investigating both transcription (by *in vitro* nuclear runoffs) and translation (by ferritin mRNA redistribution) in the same system, demonstrated an iron-induced increase in L-subunit mRNA transcription in addition to a synchronous shift of both mRNAs onto polysomes. This correlates with data obtained by protein measurement demonstrating preferential L-subunit synthesis (Kohgo *et al*, 1980). This differential synthesis of ferritin subunits provides a more complex perspective on the regulatory mechanisms.

A second component of the post-transcriptional activation of ferritin synthesis is competition of ferritin mRNA for translation. Formation of the initiation complex is the rate limiting step in the translation of many mRNA molecules. Schaefer and Theil (1981) have shown that derepressed ferritin mRNA (after induction by iron) is a strong competitor for translation initiation. A high translational efficiency of ferritn mRNA compared with other cellular messages has been demonstrated by translation in a cell-free extract (Shull and Theil, 1982). The combination of derepression of cytoplasmic stores of ferritin mRNA and high translational efficiency permits specialized iron storage cells to exhibit an extremely rapid and efficient ferritin synthetic response to iron.

A third mechanism of post-transcriptional regulation of ferritin synthesis is a variation in stability of ferritin mRNA. Steady-state levels of subunit mRNA differ between hepatoma cell lines and this has been attributed to changes in turnover of the mRNA (Costanzo *et al*, 1989). In addition, iron has been reported to increase stability of ferritin mRNA (Mattia *et al*, 1989).

1.2.5.3 Transcriptional regulation

Changes in ferritin mRNA concentration, as a result of transcriptional regulation or changes in mRNA stability have been observed in a number of situations. Theil (1987) has suggested that this is a characteristic of housekeeping ferritin, ie. ferritin produced by cells to store iron only for intracellular needs. Two cell types have been investigated, namely promyelocytic leukaemia cells (HL-60) during differentiation to neutrophils and macrophages (Chou *et al*, 1986), and adult bullfrog reticulocytes (Dickey *et al*, 1987). Differentiation of the leucocyte precursors was associated with an increase in the H/L subunit mRNA ratio, indicating a change in mRNA concentration. Total ferritin mRNA content increased transiently in macrophages but a 40-fold sustained increase was noted in neutrophils. Dickey and co-workers (1987) demonstrated that iron induces a change in ferritin mRNA concentration, by transcription or by increased mRNA stability, in situations where ferritin mRNA concentration is low, as in housekeeping ferritin in adult bullfrog red cells. This contrasts with specialized cells where ferritin mRNA is abundant and iron recruits this message for translation. Cairo *et al* (1985), studying ferritin synthesis in HeLa cells, demonstrated transcriptional increases in mRNA levels for both subunits on induction by iron, in addition to a translational change. The recent study by White and Munro (1988) discussed above suggested that transcriptional changes may also play a role in the increased L-subunit synthesis induced by iron in specialized storage cells. Rouault and co-workers (1987) showed that when changes in ferritin mRNA levels were induced artificially in an *in vitro* situation, proportional changes in ferritin biosynthetic rates were observed,

but modulation by iron was preserved. This suggests that both levels of regulation may influence ferritin synthesis synchronously.

1.2.5.4 Mechanism of translational regulation

Munro and colleagues (Zahringer et al, 1976a) first proposed that iron increases the amount of functional ferritin mRNA by removing a protein, possibly apoferritin subunits, from the 5'-end of the mRNA. The 5'-untranslated end of mRNA has been demonstrated to play a role in regulation of translation of proteins (Geballe et al, 1986; McGarry and Lindquist 1985). Sequencing of the ferritin cDNAs has demonstrated that the 5'-untranslated regions of both ferritin subunit mRNAs are unusually long compared with most eukaryotic messages. A 28 nucleotide sequence of this 5' end appears to be highly conserved in both subunits in humans (Costanzo et al, 1986; Santoro et al, 1986), as well as rats (Leibold and Munro, 1987; Murray et al, 1987), chickens (Stevens et al, 1987) and frogs (Didsbury et al, 1986). It was suggested that this conserved region may play a role in translational regulation (Murray et al, 1987).

Hentze and co-workers (1987b) first demonstrated that the 5' region of ferritin mRNA was necessary and sufficient for iron responsive regulation of ferritin synthesis. These workers subsequently identified an iron-responsive element in the human H-ferritin subunit mRNA by deletional analysis, after linking the ferritin 5'-region to a chloramphenicol acetyltransferase gene (Hentze et al, 1987a). Aziz and Munro (1987) simultaneously demonstrated this iron-responsive element occurring within the first 76 nucleotides of the 5'-untranslated region of the rat ferritin L-subunit mRNA using similar methods. Computerized modelling of the secondary structure of this region predicted the formation of a stem-loop, a configuration known

to interfere with ribosome scanning and consequently translation (Kozak, 1986).

Munro proposed that repression of ferritin translation is mediated by a negative trans-acting molecule binding to the regulatory element. In this model, activation of the mRNA in response to iron would occur by removal of this factor, allowing ribosomes to scan through the 5' region to form initiation complexes. In this context, cytoplasmic extracts from rat tissue and hepatoma cells have been shown, by electrophoretic procedures, to form RNA-protein complexes with the highly conserved sequence of the 5'-untranslated region (Leibold and Munro, 1988). The pattern of complex formation was affected by pre-treatment with iron and an 87 kD protein was identified. Rouault and co-workers (1988) subsequently demonstrated specific binding of a cytosolic protein to human ferritin H-subunit mRNA which was inhibited by a single base deletion in the iron responsive element. A correlation between the ability to interact with this cytosolic protein and the ability to function in translational regulation was demonstrated. A specific repressor of ferritin mRNA translation has been recently partially purified from rabbit reticulocyte lysate (Walden et al, 1988).

Based on sequencing of the conserved iron-responsive region, a 26 base oligonucleotide has been synthesized and was able to confer iron-responsive translation on the human growth hormone mRNA, after insertion in its 5'-untranslated region (Hentze et al, 1987a). However, the quantitative increase in response to iron was only 6-fold, whereas in the natural system ferritin synthesis increases 20- to 40-fold. Although this suggests that other regulatory processes may play a role, the iron responsive element alone has been

demonstrated to confer the complete range of iron-dependent translational regulation (Caughman et al, 1988).

Theil has shown that bullfrog ferritin mRNA is repressed when translated in a rabbit reticulocyte lysate system as compared to a wheat germ lysate and that this repression was not relieved by iron (Dickey et al, 1988). Deletion studies revealed this to be mediated by a segment of the 3'-untranslated region, implicating this region as an additional regulatory site for repression of ferritin mRNA.

Casey and colleagues (1988) have recently demonstrated that the human transferrin receptor mRNA contains five potential stem loop structures on its 3'-untranslated region, homologous to the ferritin iron responsive element. This region is necessary for iron dependent control of transferrin receptor mRNA levels, by influencing the stability of the mRNA. They propose that a common or similar regulatory molecule may control two distinct regulatory events induced by iron excess, namely the increased production of ferritin (by derepression of mRNA) and the decreased synthesis of the transferrin receptor (by decreased stability of the mRNA).

1.2.6 Turnover

The amount of ferritin in a cell is determined by both the rate of synthesis and degradation. Measurement of ferritin turnover is particularly difficult as the protein is partitioned between the cytosol and lysosomal vesicles, and very little equilibrium takes place between ferritin subunits in these cellular compartments (Theil, 1987). The half life of cellular ferritin observed in various studies is between 25 and 50 hours (Munro and Linder, 1978; Rittling and Woodworth, 1984; Treffry et al, 1984), but the lifetime of stored

iron extends to months (Green et al, 1968). This suggests that the ferritin protein coat is more stable than is indicated by pulse chase experiments (Theil, 1987).

Transient changes have been observed in rates of ferritin turnover. It has been suggested that high iron content stabilizes ferritin against breakdown and increased degradation occurs in the first few hours after induction of apoferritin synthesis by iron (Drysdale and Munro, 1966; Munro and Drysdale, 1970). Differential rates of synthesis and metabolism of the H and L subunits may complicate observations of ferritin turnover (Kohgo et al, 1980). However, this differential metabolism has not been substantiated in a subsequent study (Treffry et al, 1984) and these workers suggest that once ferritin is filled with iron, the measured turnover rate appears to be equal for the constitutive and the iron-induced ferritin synthesis.

1.2.7 Genetics

The human genome contains a number of sequences which hybridize with ferritin subunit cDNA probes. Using in situ hybridization, eight such sequences have been identified for the H-subunit on chromosomes 1, 2, 3, 6, 10, 11, 14 and X, and three for the L-subunit (Cragg et al, 1985; Lebo et al, 1985; McGill et al, 1987). The majority of these sites are probably pseudogenes as none of these sequences hybridize with an H-subunit 5'-region probe (including the TATA box through to the transcription-initiation site) and in addition they lack introns (Costanzo et al, 1986).

Functional genes have been identified by cell fusion studies, using an immune assay for detection of synthesized ferritin. Using this

method a functional H-subunit gene has been localized to chromosome 11, and the L-subunit gene to chromosome 19 (Caskey et al, 1983; Worwood et al, 1985; McGill et al, 1987). The presence of the functional genes on different chromosomes suggests that trans-acting elements may be required to coordinate expression of the subunits.

The human H-subunit gene has been characterized and shown to have three introns with four exons containing the coding region, a 208 base 5'-untranslated sequence and a 160 base 3'-untranslated region (Costanzo et al, 1986). The same gene was cloned by a different group of researchers who confirmed it to be derived from chromosome 11 and demonstrated its functionality by showing mRNA expression in transfected fibroblasts (Hentze et al, 1986). Cloning of the L-subunit gene has demonstrated a similar structure with three introns, but the localization and function of this cloned gene have not been studied (Santoro et al, 1986).

Sequencing of the ferritin subunit cDNAs (Boyd et al, 1985) has provided some insight into the genetic origin of these genes. The H- and L-subunit genes are probably derived from a common ancestor gene and diverged approximately 200 million years ago at the time of the evolutionary separation of birds and mammals. The human L-subunit cDNA has significantly more homology with the rat L-subunit than with the human H-subunit cDNA. Despite their divergence, the H and L subunits retain several common stretches of 20 to 30 nucleotides and prediction of the secondary structure of the subunit protein indicates that these genes code for similarly shaped subunits.

1.2.8 Serum Ferritin

The presence of ferritin in human serum was first demonstrated in patients with hepatocellular disease in 1956 using an immuno-precipitation technique (Reissman and Dietrich, 1956). The development of a more sensitive immunoradiometric assay resulted in the discovery that ferritin is also present in the sera of normal individuals and that the levels correlate with reticuloendothelial iron stores (Addison et al, 1972 ; Walters et al, 1973). Iron deficient patients usually have serum ferritin levels less than 10 to 12 /ug/l (Jacobs et al, 1972; Cook et al, 1976) and those with iron overload have levels over 500 /ug/l, to as high as several thousand /ug/l (Jacobs et al, 1972; Halliday et al, 1977). A number of other conditions are associated with increased serum ferritin levels. Hepatocellular disease causes release of tissue ferritin into the blood as a result of cell necrosis, leading to markedly elevated serum ferritin levels (Prieto et al, 1975). High levels are also found in patients with inflammatory diseases, associated with a low serum iron and normal or decreased total iron-binding capacity (Lipschitz et al, 1974; Birgegard et al, 1978). Neoplastic diseases such as Hodgkins disease, leukaemias (Jones et al, 1973; Parry et al, 1975) and hepatocellular carcinoma (Kew et al, 1978) are also associated with elevated levels of ferritin in the serum. Despite these limitations, measurement of serum ferritin has proved a useful clinical method of assessing body iron stores (Worwood, 1986).

The origin of serum ferritin has not been clearly defined. It has been suggested that it is synthesized by polyribosomes bound to the endoplasmic reticulum of phagocytic cells and is secreted into the blood (Bothwell et al, 1979). Serum ferritin levels have been shown

to rise after introduction of non-viable red cells, implicating the reticuloendothelial system as the source of synthesis (Siimes and Dallman, 1974). The secretory mechanism is supported by the presence of glycosylation of the serum ferritin molecule, a feature absent both from tissue ferritin and from serum ferritin associated with hepatocellular necrosis (Worwood et al, 1979). The increased serum ferritin associated with inflammatory disease is glycosylated, suggesting secretion from ferritin stores (Birgegard, 1980). Although it has been proposed that the liver is the source of serum ferritin in inflammation (Konijn et al, 1981) this has not been supported by a recent study (Schiaffonati et al, 1987). The reticuloendothelial system remains a probable source, and increased synthesis of ferritin has been demonstrated in inflammatory macrophages (Birgegard and Caro, 1984).

Circulating ferritin differs from tissue ferritin in a number of other respects. The iron content of the molecule is extremely low in comparison with tissue ferritin, even in patients with iron overload (Worwood et al, 1976). Immunologically, serum ferritin resembles liver or spleen ferritin, being rich in L-subunits as assessed by tissue specific antibodies (Worwood, 1982; Cazzola et al, 1985)). On isoelectric focusing, serum ferritin displays a wide range of isoferritins, with a pI range extending to that found in both liver (basic) and heart (acidic) ferritin (Worwood et al, 1976; McKeering et al, 1976), yet it behaves as a very basic isoferritin on anion exchange chromatography (Worwood et al, 1975). The microheterogeneity of serum ferritin appears to be due to the glycosylation of the molecule (Worwood et al, 1979), rather than variations in the ratio of H- and L-subunits. A G-subunit with molecular weight 23 000 and

which stains for carbohydrate has been described (Cragg et al, 1981). This glycosylated ferritin has a long circulating half life of approximately 30 hours (Worwood et al, 1982) compared with the rapid clearance ($t_{1/2}$ 9 minutes) of injected spleen ferritin (Cragg et al, 1983).

Ferritin rich in H-subunits has been demonstrated to occur in serum in small amounts and appears not to be directly related to the amount of storage iron (Cazzola et al, 1985). H-subunit rich ferritin has been shown to interact with serum factors to form a complex which can be demonstrated by precipitation in the presence of polyethylene glycol (Covell et al, 1984). It has been suggested that these complexes are cleared by reticuloendothelial cells, explaining the decreased proportion of H-subunit rich isoferritins in the serum. A serum ferritin-binding protein with affinity for recombinant H-subunit ferritin has recently been characterized, as a glycoprotein with molecular weight 200 000 (Bellotti et al, 1987).

1.3 THE INFLAMMATORY RESPONSE

1.3.1 Introduction

Infective, inflammatory and neoplastic processes continually challenge the host's ability to survive. The host reacts to these challenges with a number of local and systemic responses that trigger appropriate defense mechanisms. Local recruitment of phagocytes is combined with release of humoral mediators acting locally and systemically. These cytokines, released by lymphocytes and the cells of the reticuloendothelial system, include the interleukin-1 family, cachectin or tumour necrosis factor, and lymphotoxin. These inflammatory mediators have a number of overlapping biological activities. One such response is the lowering of serum iron levels, associated with an increase in storage iron. This highly conserved acute phase reaction appears to deplete the iron available for microorganisms, although in the long term it may also have certain adverse effects on the host. Macrophage and lymphocyte products involved in the acute phase response have been described for many years under a number of different names. Recent cloning of the cDNA for these proteins has helped resolve the chemical definition and biological relationships of these substances (Dinarello et al, 1986a; Pennica et al, 1985).

1.3.2 Interleukin - 1

1.3.2.1 Introduction

A number of macrophage products with various inflammatory properties have been investigated since Bennett and Beeson (1953) first described granulocyte pyrogen, a fever-inducing protein released by activated peritoneal exudate cells. An endogenously

produced circulating protein responsible for fever in experimental animals, called endogenous pyrogen, was subsequently demonstrated and it became evident that these two mediators were the same substance (Atkins et al, 1960). Kampschmidt and co-workers obtained a phagocyte product, called leukocytic endogenous mediator, which was demonstrated to produce hypoferraemia, hypozincaemia, granulocytosis and elevated serum α_2-acute phase globulins (Kampschmidt and Upchurch, 1969; Kampschmidt et al, 1973). A number of other proteins have been described with similar biological actions. Lymphocyte activating factor (Gery, 1972) and B-cell activating factor (Wood and Cameron, 1976) are released under similar conditions to endogenous pyrogen and the former has been shown to be identical to endogenous pyrogen (Murphy et al, 1980). Mononuclear cell factor (Krane et al, 1982), produced by activated monocytes, induces prostaglandin synthesis and collagenase production by synovial fibroblasts, and the protein catabolin acts as a stimulator of bone and cartilage resorption (Saklatvala et al, 1984).

The term interleukin-1 (IL-1) was proposed in 1979 to refer to the activities of lymphocyte activating factor and to include the actions of B-cell activating factor, endogenous pyrogen, leukocytic endogenous mediator and mononuclear cell factor (Aarden et al, 1979). Interleukin-1 has subsequently been purified and this protein has been demonstrated to have these multiple biological properties (Matsushima et al, 1985). Cloning of the cDNA coding for human monocyte interleukin-1 (Auron et al, 1984) has confirmed the concept of interleukin-1 as a peptide with multiple biological actions. Two forms have been cloned, IL-1-beta from human monocytes (Auron et al, 1984), and IL-1-alpha from induced HL-60 cells (Furutani et al,

1985). These two forms have also been cloned from murine cell lines (Gray et al, 1986). The two forms have only 26% sequence homology but share the multiple biological properties of IL-1. Studies employing recombinant IL-1 demonstrate that most of the acute phase response can be explained by the action of IL-1 on a variety of tissues (Dinarello, 1986; Dinarello et al, 1986a).

1.3.2.2 Production of interleukin-1

Although initial studies had focused on granulocytes as the source of inflammatory mediators, subsequent work has demonstrated the production of IL-1 by macrophages (Cebula et al, 1979) and by tissues containing mononuclear phagocytes. There is now considerable evidence that previous observation were due to contaminating macrophages in the neutrophil preparations, as purified neutrophils do not produce endogenous pyrogen or lymphocyte activating factor (Hanson et al, 1980; Dinarello et al, 1982). Phagocytic cells which have been documented to produce IL-1 include circulating blood monocytes, pulmonary alveolar macrophages, splenic macrophages (Atkins et al, 1967; Simon and Willoughby, 1981), hepatic Kupffer cells (Dinarello et al, 1968), peritoneal macrophages (Kampschmidt et al, 1983), glial cells, cutaneous Langerhans cells and renal mesangial cells (Dinarello, 1984). In addition, a number of other cell types such as corneal epithelium (Grabner et al, 1982) and epidermal keratinocytes (Sauder et al, 1982) produce IL-1.

Production of IL-1 is induced by a number of stimuli but it is not clear whether the resting mononuclear phagocyte produces this substance spontaneously (Dinarello, 1984). Microorganisms, and microbial products and antigens such as endotoxin, peptidoglycans, exotoxins, tuberculin and yeast polysaccharides stimulate IL-1

prooduction, as do inflammatory agents such as bile salts, etiocholanolone, the activated fifth complement component, and silica and urate crystals (Dinarello, 1984). Other antigens, such as penicillin and albumin, as well as plant lectins, lymphokines and drugs (eg bleomycin) may act as inducers (Dinarello, 1984).

Secretion of IL-1 appears to be activated by a membrane mediated pathway and there is good evidence that new mRNA is transcribed resulting in increased synthesis of the protein, rather than release of stored IL-1 (Dinarello and Wolff, 1982). It has been demonstrated that although no IL-1 mRNA can be detected in unstimulated monocytes, after stimulation by adhesion and exposure to endotoxin a significant amount of IL-1 mRNA is present (Auron et al, 1984).

1.3.2.3 Biological actions

Interleukin-1 acts on a number of tissues and organs to produce a variety of physiological effects. The precise mechanism of action has not been elucidated, but an 80 000 dalton receptor has been identified on a number of IL-1 responsive cells (Dower et al, 1985). The calcium-phosphoinositol pathway has been suggested as a second messenger system and it is likely that many of the growth promoting effects of IL-1 are mediated by leukotrienes (Dinarello, 1988). The biological effects of IL-1 appear to be the same for both the alpha and the beta form (Dinarello, 1986) and are as follows:

Fever: This is associated with increased hypothalamic concentrations of prostaglandin E (Bernheim et al, 1980) as well as increased central concentrations of endorphins (Murphy et al, 1983).

Hepatic protein synthesis: Hepatocytes are induced to synthesize a number of acute phase proteins, namely serum amyloid A, C-reactive protein, complement components, fibrinogen, caeruloplasmin and

haptoglobin (Dinarello, 1988). Albumin, transferrin and fibronectin levels decrease (Kushner, 1982) and IL-1 depresses the activity of liver cytochrome P-450 dependent drug metabolism (Ghezzi et al, 1986). IL-1 regulates the synthesis of these proteins at the level of transcription of mRNA (Ramadori et al, 1985).

Decreased serum levels of iron and zinc: Interleukin-1 causes hypoferraemia (Kampschmidt and Upchurch, 1969) associated with an increase in hepatic iron storage (Pekarek et al, 1972) but the mechanism has not been clearly defined. This aspect is discussed in more detail in a subsequent section. Plasma zinc levels also decrease (Pekarek et al, 1972) and this occurs as a direct effect, independent of granulocytes (Goldblum et al, 1987).

Granulocytosis: When injected into rats, IL-1 induces a neutrophilia (Kampschmidt et al, 1972), with an egress of mature neutrophils from the bone marrow. Repeated injections cause a sustained granulocytosis associated with increase granulopoiesis (Kampschmidt and Upchurch, 1977). This increased production of granulocytes by the bone marrow may occur as a result of release of granulocyte-macrophage colony stimulating factor from endothelial cells (Zucali et al, 1986). In addition, IL-1 induces oxidative metabolism in granulocytes, and stimulates exocytosis of the lysozyme and lactoferrin-containing secondary granules from neutrophils (Klempner et al, 1978 & 1979).

Immunoregulatory effects: Studies using recombinant IL-1 have confirmed the importance of this protein in T-cell activation (Williams et al, 1985). IL-1 induces the production of interleukin-2 by T-lymphocytes as well as expression of the interleukin-2 receptor by these cells. IL-1 also acts as a cofactor in the activation of B-cells, inducing antibody production (Muraguchi et al, 1984). It has

been shown that IL-1 induces production of a B-cell stimulating factor, which has recently been demonstrated to be interferon-β 2/interleukin-6 (Van Damme et al, 1987).

Catabolic effects: IL-1 acts as an ostecolast-activating factor, producing local resorption of bone and cartilage (Saklatvala et al, 1985). Systemically it produces muscle proteolysis which may be mediated by prostaglandin E_2 (Baracos et al, 1983). Systemic catabolic effects may also occur as a results of increased cortisol secretion (Besedovsky et al, 1986).

Endothelial cell activation: Endothelial cells are activated to induce release of the vasodilatory prostaglandins I_2 and E_2, and to alter the endothelial plasma membrane to increase the adhesion of neutrophils and monocytes (Rossi et al, 1985; Bevilacqua et al, 1985). Endothelial cell surface procoagulant activity is enhanced (Dejana et al, 1987) and IL-1 induces localized vasculitic changes by chemotactic effects (Hunninghake et al, 1987), production of a plasminogen-activator inhibitor (Nachman et al, 1986) and release of platelet-activating factor (Dejana et al, 1987).

Effects on fibroblasts: IL-1 is necessary for the production of granulomas (Kobayashi et al, 1985) and directly increases the production of collagen by fibroblasts (Canalis, 1986).

CNS effects: In addition to fever, IL-1 acts centrally to induce slow-wave sleep (Kreuger et al, 1984) and may cause the loss of appetite associated with inflammatory disease (Dinarello, 1988).

1.3.3 Tumour Necrosis Factor

1.3.3.1 Introduction

Tumour necrosis factor (TNF) or cachectin is a cytokine produced by macrophages, which mediates a number of biological events associated with infectious and neoplastic disease. Two independent routes of investigation resulted in isolation of this substance. Studies of the mechanisms of cachexia in chronic disease resulted in the isolation of a polypeptide hormone, cachectin, which suppressed lipoprotein lipase activity (Beutler et al, 1985b). Other workers, investigating the phenomenon of tumour necrosis associated with bacterial infections described by Coley in 1893 (Beutler and Cerami, 1987), isolated a serum factor from endotoxin-injected animals (Carswell et al, 1975). Purification of this tumour necrosis factor was achieved in 1985 (Aggarwal et al, 1985) and it was noted that it had strikingly similar amino acid sequences to cachectin (Beutler et al, 1985). Subsequent cDNA sequencing has confirmed that TNF and cachectin bioactivities derive from the same protein (Pennica et al, 1985; Caput et al, 1986). The protein is produced as a precursor peptide which is cleaved to its mature form of 157 amino acids (Beutler and Cerami, 1986) and is secreted as a trimer of 3 identical subunits (Smith and Baglioni, 1987). This macrophage-derived TNF/cachectin has now been called TNF-alpha to differentiate it from the lymphoid derived TNF-beta, previously known as lymphotoxin (Aggarwal et al, 1987). These two proteins have 52% sequence homology, both have genes on chromosome 6 and both bind to the same high affinity receptor. In addition, they have similar biological actions when examined in the same system (Nathan, 1987).

TNF is secreted by macrophages and by mononuclear phagocytes from various tissues (Mannel et al, 1980; Matthews, 1981) as well as T-lymphocytes and Epstein-Barr virus-transformed B-cell lines (Williamson et al, 1983; Cuturi et al, 1987). Secretion is stimulated by endotoxin, and may occur in large quantities, accounting for 1 to 2 % of the total protein secreted by murine macrophage cell lines (Beutler et al, 1985b). Other unrelated substances such as muramyl dipeptide, viruses and mitogens also induce TNF production (Gifford and Flick, 1987). Glucocorticoids and prostaglandin E_2 inhibit synthesis of TNF. Circulating monocytes produce only small amounts of TNF, but synthesis can be increased by priming with gamma-interferon (Beutler and Cerami, 1987).

1.3.3.2 Biological actions

TNF has a wide spectrum of biological activities, but the mechanism of action of this hormone has not been elucidated. TNF acts via a high affinity receptor on a number of tissues to regulate the transcription of various proteins. The post-receptor mechanisms are not known, but may be similar to interleukin-1 and some of its actions are known to be mediated by prostaglandins and leukotrienes (Fiers et al, 1987).

Haemorrhagic necrosis of tumours was first noted in the late 19th century, and although the mechanism is still not entirely clear, it appears to arise through vascular events. TNF stimulates endothelial procoagulant activity and inhibits thrombomodulin expression, resulting in thrombosis in tumour vessels (Nawroth and Stern, 1986).

TNF plays a major role in the septic shock syndrome. The complex haemodynamic and metabolic changes occurring during endotoxaemia appear to be mediated by TNF. Infusion of TNF into rats precipitates

a metabolic acidosis, tachypnoea, hypotension and shock (Tracey et al, 1986). These effects are most probably a result of an endothelial lesion allowing fluid exudation into the extravascular space (Beutler and Cerami, 1987). Increased permeability of the skeletal muscle plasma membrane contributes to this effect (Tracey et al, 1987). Leukotriene and platelet-activating factor release may be stimulated by cachectin and these secondary mediators may cause the polymorphonuclear leukocyte infiltration found in numerous organs (Beutler and Cerami, 1987). A number of metabolic effects have been described including hyperglycaemia, hyperkalaemia and metabolic acidosis, probably occurring as secondary phenomena (Tracey et al, 1986). TNF has been implicated in the pathogenesis of malaria and in the early changes of the graft versus host reaction (Beutler and Cerami, 1988).

TNF acts on adipocytes causing the cachexia of chronic disease. This it does by inhibiting lipoprotein lipase (Beutler et al, 1985b), reducing triglyceride uptake. It also prevents differentiation of pre-adipocytes and inhibits transcription of several lipogenic enzymes (Tracey et al, 1987).

In addition to the above actions, TNF has a number of functions in common with interleukin-1. These include endothelial activation resulting in increased procoagulant activity and neutrophil adherence (Pober, 1987); bone and cartilage resorption by stimulating production of collagenases and prostaglandin E_2 (Bertolini et al, 1986); endogenous pyrogen activity, both directly and by inducing interleukin-1 release (Dinarello et al, 1986b); and induction of granulocyte-macrophage colony stimulating factor production by a variety of cell types (Munker et al, 1986). Induction of hepatic

acute-phase protein synthesis is another function shared with interleukin-1 (Perlmutter et al, 1986), although the extent to which TNF contributes to this response is not known. Recently, TNF has been demonstrated to increase ferritin H-subunit synthesis, producing this effect at a transcriptional level, increasing biosynthesis of ferritin mRNA (Torti et al, 1988)

TNF has some beneficial antimicrobial actions. Antiviral activity has been demonstrated (Mestan et al, 1986) and enhanced immune cell function and phagocytosis occur (Shalaby, 1985). TNF has been shown to have antimalarial activity (Beutler and Cerami, 1987), and it enhances eosinophil toxicity to Schistosoma mansoni (Silberstein and David, 1986). It has also been suggested that even in the absence of a survival advantage to the individual, TNF may benefit the population as a whole by rapidly removing infected individuals (Beutler and Cerami, 1988).

1.3.4 Interleukin-6

Interleukin-6 (IL-6) is the name given to describe the cytokine previously known as interferon-β_2, B-cell stimulating factor 2 (BSF-2) and hepatocyte stimulating factor (Dinarello et al, 1989). This multifunctional secretory glycoprotein plays an important role in the host inflammatory response and has a wide spectrum of actions. It is only recently that these various stimulatory factors have been demonstrated to be the same cytokine. The biological actions attributed to IL-6 include induction of acute phase protein synthesis, endogenous pyrogen activity, activation of B- and T-lymphocytes, stimulation of immunoglobulin synthesis and anti-viral activity (Sehgal et al, 1987; Dinarello, 1989).

The major source of IL-6 is the mononuclear phagocyte (Bauer et al, 1988) but it is also produced by fibroblasts, T-cells, endothelial cells and keratinocytes (Helfgott et al, 1987; Sehgal et al, 1988). IL-6 production is enhanced in response to other cytokines such as IL-1, TNF, platelet-derived growth factor and other interferons (Sehgal et al, 1987; Ray et al, 1988). Bacterial products such as lipopolysaccharide and viral pathogens stimulate IL-6 synthesis (Helfgott et al, 1987; Sehgal et al, 1988).

IL-6 plays an important role in acute phase protein synthesis by the liver (Gauldie et al, 1987; Sehgal et al, 1988). The stimulation of hepatic protein synthesis demonstrated by IL-1 may be mediated in part by IL-6, although both cytokines appear to be active individually (Moshage et al, 1988). In addition to acute phase protein synthesis, IL-6 may be responsible for decreased synthesis of the "negative acute phase proteins" such as albumin (Andus et al, 1987). In the clinical situation, high serum levels of IL-6 have been demonstrated in patients with acute inflammation, correlating with the degree of fever (Nijsten et al, 1987). No data yet exists on the possible role of IL-6 in the induction of ferritin synthesis in inflammation.

1.3.5 The acute phase response

The acute response to inflammatory, infective, traumatic or neoplastic injury results in metabolic and physiologic changes aimed at containing or destroying infectious agents, removing or repairing damaged tissue, and permitting the organism to survive. Locally, changes in vascular tone and permeability occur, associated with chemotaxis of leucocytes. At the same time, a number of systemic

effects take place, largely as a result of release of cytokines. Three substances have been implicated as being responsible for these effects, namely IL-1, TNF and IL-6. This acute phase response constitutes an array of changes, some of which have known beneficial value to the host, but in many cases a useful function is not readily apparent and some effects may be deleterious (Kushner, 1982).

The metabolic changes that occur include protein catabolism and increased gluconeogenesis (Beisel, 1977), while variable changes in lipid metabolism have been described (Masoro, 1977). Increased synthesis of a number of hormones including glucagon, insulin, ACTH, cortisol, catecholamines, thyroxine and growth hormone have been shown to occur (Beisel, 1977). Immunosuppression has been observed following surgical inflammation and a number of possible mechanisms have been postulated (Kushner, 1982). The various effects of IL-1, TNF and IL-6 including fever, alterations in serum iron, copper and zinc levels, and changes in hepatic protein synthesis have been discussed above.

The alteration in hepatic synthesis of a number of proteins is an important component of the acute phase response. The magnitude and time of onset of the rise in serum levels varies between proteins (Aronsen et al, 1972). C-reactive protein and serum amyloid A-protein generally increase several hundred fold, while others such as fibrinogen, α_1-acid glycoprotein, α_1-antitrypsin, α_1-antichymotrypsin, haptoglobin, caeruloplasmin and the third complement component rise only 2 to 4 fold (Kushner, 1982). In contrast, a number of proteins exhibit a fall in serum levels during the acute phase. These so-called "negative acute phase reactants" include albumin, transferrin and fibronectin (Kushner, 1982). The mechanism

of fall may reflect diminished hepatic synthesis or increased catabolism (Kushner, 1982; Aisen, 1984). Interleukin-1, tumour necrosis factor and interleukin-6 may all play a role in mediating this response.

Serum ferritin exhibits the behaviour of an acute phase protein (Konijn and Hershko, 1977), but whether this occurs as a primary event due to increased hepatic synthesis, or merely reflects the increased reticuloendothelial ferritin stores accompanying inflammation, has not been elucidated. Recent work suggests that increased hepatic secretion of ferritin does not occur in experimental inflammation (Schiaffonati et al, 1988). The mechanism of ferritin synthesis in inflammation is discussed in more detail in section 1.4.4 below.

1.4 IRON AND INFLAMMATION

1.4.1 Microorganisms and iron

With the possible exception of Lactobacillus, all microorganisms require iron for growth (Griffiths, 1987). Host iron is predominantly intracellular or bound to iron transport proteins, making it poorly accessible to microorganisms, which require iron at a concentration of 0,4 to 4 $x10^{-5}$ M (Barclay, 1985). The free iron available is 10^{8}-fold less than that required for microbial growth (Weinberg, 1975) and the availability of iron therefore affects the growth and invasive potential of numerous pathogens. These include Escherichia coli (Bullen and Rogers, 1969), Listeria monocytogenes (Sword, 1966), Neisseria species (Holbein et al, 1979), Yersinia enterocolitica (Robins-Browne and Prpic 1985), Mycobacterium tuberculosis (Kochan, 1973), Legionella pneumophila (Quinn and Weinberg, 1988), Plasmodium falciparum (Pollack, 1983) and numerous others (Griffiths, 1987). A number of virulence factors are also iron dependent, such as production of toxin A by Pseudomonas aeruginosa (Bjorn et al, 1978) and diphtheria toxin by Corynebacterium diphtheriae (Pappenheimer, 1977).

Microorganisms have therefore developed mechanisms in order to acquire iron. The commonest of these is the production of siderophores, either hydroxamates or phenolates (catechols) (Barclay, 1985). These genetically determined factors are synthesised and exported, assimilating iron from the environment and returning it to the microorganism via specific outer membrane protein receptors. Pathogenic fungi have also been demonstrated to produce hydroxamate siderophores (Holzberg and Artis, 1983). In addition to their necessity for growth, the virulence of Vibrio species (Crosa et al,

1977) and Salmonella typhimurium (Hoiseth and Stocker, 1981) are related to the presence of siderophores.

Pathogenic Neisseria species have been demonstrated to obtain iron directly from transferrin, independent of siderophores, by a saturable cell surface mechanism (Mickelsen and Sparling, 1981; Simonson et al, 1982). When iron is limited, these organisms express specific outer membrane proteins, which are repressed in the presence of transferrin, lactoferrin and to a lesser extent haemoglobin (West and Sparling, 1985). Plasmid-mediated iron sequestering systems occur and have been demonstrated to play a role in conferring virulence to bacteria (Crosa, 1980; Williams, 1979; Stuart et al, 1980). A number of other mechanisms for obtaining iron have been described. Proteolytic degradation of haemoglobin, haptoglobin and haemopexin occurs, demonstrated by Bacteroides (Carlsson et al, 1984) and Escherichia coli (Bornside et al, 1968). Listeria monocytogenes secretes a soluble reductant which converts Fe^{3+} to Fe^{2+} allowing release of iron from the iron-transferrin complex (Cowart and Foster, 1985). Protozoans such as Entamoeba, Naegleria, and Plasmodium require iron but no siderophores have been found (Weinberg, 1984). Plasmodium falciparum appears not to use the abundant haem iron in the red cell, but takes up transferrin iron, possibly by receptors that it inserts into the erythrocyte membrane (Pollack and Fleming, 1984).

The presence of host defences preventing microorganisms from acquiring iron was first demonstrated by Schade and Caroline (1944) who serendipidously found an inhibitory factor (conalbumin or ovotransferrin) in egg white. The term "nutritional immunity" has been used to emphasize the importance of iron deprivation in limiting

the growth of invading microorganisms (Kochan, 1973). The avid binding of iron by transferrin in the serum makes it poorly available to microorganisms (Bullen, 1981) although as mentioned, some organisms have the ability to take up transferrin iron. Lactoferrin is a protein found in milk, secretions, plasma and neutrophils. It has a high affinity for iron at a low pH and its iron-repressable antimicrobial activity has been demonstrated against a number of organisms (Arnold et al, 1977 & 1980). Antibodies to siderphores, in particular enterochelin produced by E.coli and S.typhimurium, have been found in normal human serum and may contribute to the iron-reversible bacteriostatic effect of serum (Moore et al, 1980). Hypoferraemia due to impaired release of reticuloendothelial iron is a feature of inflammatory diseases and it is possible that this may play a host defence role. This mechanism is discussed in detail in the following sections.

That pathogenic bacteria experience iron-restricted conditions in vivo is demonstrated by their expression of certain outer membrane proteins, which also appear when grown in iron deficient media but are repressed in iron sufficient media (Lam et al, 1984; Brown et al, 1984). The importance of "nutritional immunity" is dramatically demonstrated by the severe invasive mucormycosis occurring in diabetic ketoacidosis. The low pH makes transferrin unable to withhold iron from the Rhizopus organism, resulting in unusually excessive growth (Artis et al, 1982).

Other trace metals may also be involved in nutritional immunity. Copper deficiency in mice results in decreased synthesis of caeruloplasmin, which acts in the plasma as a ferroxidase. As a result, the return of reticuloendothelial iron to the transferrin

pool is inhibited, protecting from Neisserial infection (Letendre and Holbein, 1984a). The decrease in serum zinc levels which occurs in inflammation has been suggested to play a similar role in depriving microrganisms of this nutient (Sugarman, 1983).

1.4.2 Iron metabolism in inflammation

Inflammatory states are associated with a number of characteristic changes in iron metabolism. Freireich and co-workers (1957) first demonstrated decreased recycling of haemoglobin iron associated with a decreased plasma iron level in experimental animals with induced inflammation. Decreased iron release from the reticulo-endothelial system was subsequently demonstrated in patients with chronic inflammation (Noyes et al, 1960). As discussed in section 1.1, iron release from the reticuloendothelial system following administration of heat-damaged red cells occurs with a biphasic rise in serum iron. Endotoxin administration to rats results in hypoferraemia, beginning at 4 hours with a nadir at 12 hours (Kampschmidt and Schultz, 1961) and the iron appears to be shifted from the early release pool to the late release pool (Fillet et al, 1974). Hershko and co-workers (1974) using turpentine-treated rats demonstrated a decreased plasma iron turnover, decreased iron absorption and a decrease in the early release pool (from 72% in control rats to 38%). The decrease in early release of iron may be due to a decrease in the rate of haem degradation or an increase in incorporation of iron into ferritin stores. Both mechanisms appear to play a role and the increased incorporation into ferritin can be partially overcome by stimulation of iron release by inducing iron deficiency (Torrance et al, 1978).

The so-called "reticuloendothelial iron block" results in a number of measurable changes in iron parameters. These are a reduced serum iron concentration, a reduced total iron-binding capacity and a raised serum ferritin concentration. In addition to reticulo-endothelial cells, intestinal mucosal cells are affected, resulting in decreased absorption of iron (Torrance et al, 1966; Beresford et al, 1971). The anaemia of chronic disorder may follow, due to iron deficient erythropoiesis as evidenced by decreased marrow sideroblasts and an increase in free erythrocyte protoporphyrin (Lee, 1983). However, the role of iron deficient erythropoiesis in this anaemia has been questioned (Baynes et al, 1986b).

The abnormalities described have been confirmed in numerous clinical studies in addition to animal models. Decreased serum iron associated with increased serum ferritin concentration has been observed in aseptic inflammation induced by endotoxin or etiocholanolone in human volunteers (Elin et al, 1977). A longitudinal study of infectious illnesses demonstrated similar changes, persisting for several weeks (Birgegard et al, 1978). Disturbances of iron metabolism have also been noted in chronic rheumatoid arthritis (Baynes et al, 1987), acute myocardial infarction (Griffith et al, 1985) and severe sustained exercise (Taylor et al, 1987).

The increase in serum ferritin occurring in such situations has been demonstrated to be due to glycosylated ferritin, characteristic of synthesized and released ferritin (Birgegard, 1980). This rise in serum ferritin appears therefore to be a function of the increase in ferritin stores (Cook et al, 1974; Bezwoda et al, 1979), rather than release by damaged cells.

1.4.3 Mechanisms of the hypoferraemic response

The underlying mechanism of the hypoferraemic response to inflammation has not been clearly elucidated and a number of theories have been proposed. Controversy arises as to whether the increase in ferritin production in reticuloendothelial cells occurs as a primary event or whether it is secondary to a rise in intracellular iron levels.

In favour of a primary rise in ferritin synthesis is the work of Konijn and colleagues (1977), who showed that the increase in hepatic and splenic synthesis of ferritin in inflammation occurs significantly earlier than the changes in plasma iron. They suggested that ferritin synthesis increased as an acute phase reactant, probably in response to increased serum levels of interleukin-1. However this temporal relationship may be difficult to interpret as small changes in intracellular iron levels may occur without a measureable variation in plasma iron. In addition there is no evidence that interleukin-1 directly induces ferritin synthesis in reticuloendothelial cells.

The fact that iron deficiency limits the ferritin synthetic response in inflammation (Lipshitz et al, 1974) seems to indicate that changes in intracellular iron levels may be of primary importance. Lactoferrin is an iron-binding glycoprotein with a molecular weight of 75 000 to 80 000 daltons, which is widely distributed in body fluids and tissues, and has been implicated as playing a role in the hypoferraemic response. Lactoferrin is released by neutrophils in response to interleukin-1 (Klempner et al, 1978) and neutrophils have been suggested as being necessary for the hypoferraemia of inflammation (Kampschmidt and Upchurch, 1969).

Although the methods used to deplete neutrophils in the latter study may have inhibited hypoferraemia by other mechanisms, a recent study investigating the hypoferraemia induced by interleukin-1 demonstrated that this change in serum iron is dependent on the presence of neutrophils (Goldblum et al, 1987). Van Snick and co-workers (1974) proposed that lactoferrin released by inflammation scavenges plasma iron from transferrin or from microorganisms, transferring it to reticuloendothelial cells. Increasing levels of intracellular iron would secondarily stimulate ferritin synthesis in these reticuloendothelial cells. Lactoferrin has a high affinity for iron, and has been demonstrated to remove iron from transferrin at pH < 7,0 when given in supraphysiological doses (Van Snick et al, 1974). In further support, mouse peritoneal macrophages (Van Snick et al, 1976), alveolar macrophages (Markowetz et al, 1979) and human monocytes (Birgens et al, 1983) have been shown to have surface receptors for lactoferrin. The ingestion of lactoferrin by monocytes and macrophages, and transfer of its iron to ferritin has also been demonstrated (Van Snick et al, 1977; Birgens et al, 1988). The kinetic studies of Bennett and Kokocinski (1979) showed that administered lactoferrin is avidly sequestered by liver and spleen and its iron is only slowly made available for erythropoiesis. However, a physiological transport role for lactoferrin has yet to be clearly demonstrated and the concentration of lactoferrin in the plasma is extremely low, being two orders of magnitude lower than transferrin (Taylor et al, 1987). Longitudinal clinical data comparing acute phase markers with lactoferrin concentration do not support the concept that this protein is responsible for the hypoferraemia of inflammation (Baynes et al, 1986a).

A second possible mechanism of increasing intracellular reticuloendothelial iron is direct uptake of transferrin iron (O'Shea et al, 1973). In the normal situation macrophages obtain little, if any, iron from transferrin and transferrin receptor expression is decreased or absent (Andreesen et al, 1984). Binding of transferrin iron by macrophages has been demonstrated in vitro, with increased transferrin uptake by macrophages activated by acute inflammation (MacDonald et al, 1969; Wyllie, 1977), as well as increased transferrin receptor expression (Hamilton et al, 1984). Peritoneal macrophages activated by acute inflammation have been shown to have an increased ability to take up iron (Birgegard and Caro, 1984). A serum factor which enhances transferrin uptake by human macrophages in vitro has been demonstrated in inflammation (Baynes et al, 1988). Nevertheless, ferrokinetic studies show that reticuloendothelial uptake of transferrin iron does not usually play a significant role in iron turnover, even in infection (Finch et al, 1970).

Also against the concept of increased reticuloendothelial uptake of plasma iron are the kinetic studies of Letendre and Holbein (1983 & 1984). Using mice infected with Neisseria meningitidis, they demonstrated impaired reticuloendothelial release of iron but normal turnover of transferrin iron. No increase in uptake of labelled tranferrin iron by the reticuloendothelial system was noted.

Other possible mechanisms for the hypoferraemia of inflammation have been suggested. A primary membrane abnormality may occur, inhibiting transport of iron from the reticuloendothelial cell. Mechanisms of release of iron are however very poorly understood. Increased production of heamosiderin, due to digestion of the ferritin protein shell by lysosomal enzymes, may result in

partitioning of iron in this unavailable compartment (Roeser, 1980). Hershko and co-workers (1974) have demonstrated increased hepatic haemosiderin production in turpentine-treated rats.

1.4.4 Ferritin synthesis in inflammation

Increased ferritin synthesis plays an important role in the hypoferraemic response, whether as a primary or secondary event. Torrance and co-workers (1978) demonstrated an increased incorporation of iron in ferritin in an endotoxin-treated rat model and it has been shown that macrophages from mice with inflammation exhibit increased ferritin synthesis and increased iron uptake (Birgegard and Caro, 1984). In humans, Summers and Jacobs (1976) observed an increased ferritin content in peripheral blood monocytes of patients with the anaemia of chronic disease, and similar results have been obtained in patients with acute inflammation (Lipschitz et al, 1975).

Konijn and co-workers (1977) studied ferritin synthesis in the livers and spleens of rats with turpentine-induced inflammation. Ferritin synthesis measured by ^{3}H-leucine incorporation increased to twice normal in the liver and spleen by 4 hours after injection. This preceeded the changes in plasma iron turnover, the maximal reduction of plasma iron occurring at 12 hours. Since iron-induced ferritin synthesis occurs at 5 hours following iron injection (Drysdale and Munro, 1966), these authors postulate that the increased ferritin synthesis in inflammation must occur as a primary event.

In a subsequent study, the respective roles in ferritin synthesis of "cell sap" (a post-mitochondrial supernatant) factors and polysomes, were investigated in hepatic tissue (Konijn et al, 1981).

Two waves of increased ferritin synthesis were noted. The early wave, peaking at 6 hours, was associated with increased activity of "cell sap" factors, based on phenotypic expression using *in vitro* translation. This could be due to either derepressed ferritin mRNA or increased relative amounts of ferritin mRNA. The late phase, starting at 24 hours and peaking at 48 hours, resulted in a greater increase in ferritin synthesis than the first phase, combined with an increase in total protein synthesis. Both "cell sap" and polysomes demonstrated increased synthetic activity, interpreted as combined transcriptional and translational activity.

Cloning of cDNA for human and rat ferritin subunits (Boyd *et al*, 1985; Brown *et al*, 1983) has allowed direct assessment of mRNA content and distribution by hybridization. A recent study by Schiaffonati and colleagues (1988) investigated liver ferritin synthesis in the turpentine injected rat. No increase in total L-subunit mRNA was found although inflammation stimulated L-subunit protein synthesis preferentially. Splenic ferritin synthesis was not investigated, and probing for the H-subunit was not performed. In terms of assessing the source of secreted serum ferritin, their data demonstrates that the increased serum ferritin in inflammation does not originate in the liver. In addition, the amount of L-subunit mRNA attached to free and membrane-bound ribosomes was studied. No increase in either fraction was noted, but the authors maintain that this is compatible with translational regulation as the total amount of bound polysomes increases in inflammatory states (Piccoletti *et al*, 1984). The distribution of free to polysome-bound mRNA was not studied.

1.4.5 Clinical Implications

1.4.5.1 Infection and iron status

The "nutritional immunity" hypothesis suggests that iron overload should lead to an impairment of protection against infection while iron deficiency reduces the risk. In chronic iron overload, transferrin saturation is high and significant amounts of iron may appear in the plasma as non transferrin-bound iron, being readily available to bacteria (Batey et al, 1980; Hershko et al, 1988). Low molecular weight complexes of iron have been demonstrated in the serum of patients with idiopathic haemochromatosis (Gutteridge et al, 1985) and increased susceptibility to infection may occur in this condition (Weinberg, 1978). Yersinia enterocolitica, a microorganism unable to produce a siderophore, may thrive in such situations of iron excess or treatment with the exogenous siderophore desferrioxamine (Rabson et al, 1975; Robins-Browne and Prpic, 1985). Vibrio vulnificus may also use this drug to obtain iron, resulting in infection (Wright et al, 1981). However, clinical evidence of increased incidence or severity of infection may well be affected by a number of interfering variables. Advanced liver disease itself may predispose to increased infection (Marlon et al, 1971) and the high incidence of bacterial infection in secondary haemosiderosis may be due to associated functional asplenia or previous splenectomy (Smith et al, 1964; Bullen and Losowsky, 1979). Patients with sickle cell anaemia have large amounts of free haemoglobin in their plasma (Nauman et al, 1981) and this may play a role in their increased susceptibility to infection (Barrett-Connor, 1971). A similar mechanism may cause an increase in bacterial infections in patients with malaria (Kluger and Bullen, 1987).

Studies investigating the susceptibility of iron deficient patients to infection are complicated by the occurrence of the hypoferraemia of inflammation. Masawe and co-workers (1974) studied 110 anaemic patients in Tanzania and found a decreased incidence of bacterial infections but an increased incidence of malaria in an iron deficient group compared with other anaemic patients. As no comparison with normal controls was made, it is possible that the non iron deficient anaemic patients may have had an increased incidence of infections due to the underlying haematologic disease. In contrast, an increased incidence of chronic mucocutaneous candidiasis has been demonstrated to be associated with iron deficiency (Higgs and Wells, 1972).

1.4.5.3 Infection and iron therapy

Several studies have investigated the effect of iron replacement on infection. Prophylactic parenteral iron given soon after birth has been reported to increase the incidence of neonatal infection (Barry and Reeve, 1977; Oppenheimer et al, 1986) although other studies have shown no increase in susceptibility to infection (Leiken, 1960) or decreased infections (Hershko et al, 1988). Oral iron supplementation has been demonstrated to be associated with serious adverse effects in malnourished African children. A direct correlation between initial serum transferrin concentration and survival after treatment in kwashiorkor has been described (MacFarlane et al, 1970) and the combination of iron replacement therapy in the presence of low iron binding capacity may provide free iron for bacterial consumption. However, those patients with the low total iron binding capacity had more severe protein malnutrition and death may have been independent of iron treatment. Murray and co-workers (1978) studied anaemic Somali nomads treated with iron. A dramatic increase in episodes of

infection occurred in the treated group compared with untreated controls, particularly with respect to malaria, brucellosis and tuberculosis.

The hypoferraemia of inflammation differs from iron deficiency in many important respects. Iron deficiency is associated with a decrease in the amount of iron available for enzymes and oxygen transport systems. In this situation, the potential beneficial effects of the nutritional immunity may be offset by interference with other host defence mechanisms. However, the hypoferraemia of inflammation may have the advantages of reduced iron availability for microorganisms without the adverse effects. Whether correction of the hypoferraemia of inflammation aggravates existing infections has not been studied directly (Hershko et al, 1988).

1.4.5.3 Anaemia of chronic disorder

An important association with the hypoferraemic response is the anaemia of chronic disease. This has been defined as an anaemia associated with chronic infective, inflammatory, traumatic or neoplastic disease that occurs in the presence of the characteristic disturbances of iron metabolism accompanying inflammation (Lee, 1983). It is an important cause of anaemia, particularly in elderly males (Dallman et al, 1984). The degree of anaemia is usually mild and non-progressive (Cartwright, 1966) and symptoms are few, often only those of the underlying disease.

The reticuloendothelial block to iron release associated with inflammation may depress erythropoiesis by limiting iron availability (Douglas and Adamson, 1975) but the importance of this mechanism has been questioned (Cavill and Bentley, 1982; Baynes et al, 1986b). A number of other factors play a role in the mechanism of this anaemia.

Decreased red cell survival occurs (Cavill et al, 1977), due to the increased phagocytic activity of activated macrophages (Cartwright and Lee, 1971), or as a direct effect of fever (Karle, 1974). A reduced erythropoietin response has also been implicated (Douglas and Adamson, 1975). Interleukin-1 appears to play a role in a number of these mechanisms and is probably the common pathogenetic factor linking the various diseases associated with the anaemia of chronic disorders.

2. MATERIALS AND METHODS

2.1 INTRODUCTION

The experimental methods required for investigation of ferritin mRNA in inflammatory states involved largely molecular biological techniques not previously used in this laboratory. As a result, numerous methodologies were developed, including the extraction, electrophoresis and blotting of ribonucleic acid; plasmid deoxyribonucleic acid transformation into bacteria, and extraction and purification from bacterial culture; restriction digestion; radiolabelling of nucleic acid; and hybridization. Standard texts such as that of Maniatis and colleagues (1982), and Davis and co-authors (1986) provided invaluable practical information and a number of newer methods were also investigated and applied (Chomczynski and Sacchi, 1987; Chung and Miller, 1988). The assistance of Dr. G. Car, as well as colleagues in the Departments of Microbiology and Biochemistry, University of Cape Town and in the Department of Biochemistry, University of the Witwatersrand is appreciated. Numerous technical problems were nevertheless encountered and resolved, providing a most valuable insight into scientific methodology.

2.2 MONOCYTE PREPARATION

2.2.1 Mononuclear separation and monocyte purification

Peripheral blood monocytes, being a representative reticulo-endothelial cell studied, were fractionated from whole blood. As the number of monocytes available from a single subject is limited by the amount of blood removed, an efficient method of purification was developed. Mononuclear cells (monocytes and lymphocytes) can be easily separated from whole blood by centrifugation on Ficoll-hypaque

(Boyum, 1968). Several methods have been described for purification of monocytes from this mononuclear preparation, of which adhesion to culture dishes is the simplest and commonest technique and was the method eventually used. The "panning" method of Wysocki and Sato (1978) is attractive in that it is technically simple, is highly specific but is expensive and results in a relatively low yield of monocytes. Other methods for monocyte separation are density gradient centrifugation in Percoll (Feige et al, 1982) and centrifugal elutriation (Mason and Weiner, 1985). These methods are reported to produce a purer monocyte preparation, but the yield is not better than adherence and large volumes of blood are required.

Mononuclear cells were separated from whole blood using the method of Boyum (1968). Heparinized blood (100 ml) was diluted in an equal volume of 0,9% (w/v) saline and 33 ml was layered onto 17 ml Ficoll-hypaque (Pharmacia, Uppsala, Sweden) in a sterile 50 ml polypropylene tube. The 6 tubes were centrifuged at 400 g for 35 minutes at 4° C and the mononuclear cells were harvested from the interface between the Ficoll-hypaque and plasma. The cells were washed in phosphate-buffered saline (PBS) and red cells lysed by incubation on ice in NH_4Cl (155 mM, with $NaHCO_3$ 10 mM, EDTA 0,1 mM). The cells were washed and resuspended in 20 ml PBS.

Three methods of monocyte purification were assessed, namely adhesion to polystyrene Petri dishes (Rabinowitz, 1964; Brodersen and Burns, 1973), adhesion using foetal calf serum coated polystyrene Petri dishes (Kumagai et al, 1979), and an antibody "panning" technique (Wysocki and Sato, 1978). For adhesion, 4 ml aliquots of mononuclear cell suspension were added to the Petri dishes (90 mm diameter) and incubated at 37°C for 70 minutes. The non-adherent

cells were removed by washing 3 times with PBS. A similar procedure was followed using Petri dishes coated with heat-inactivated foetal calf serum (FCS). The "panning" method used mouse IgM monoclonal antibody to the monocyte antigen Mo2 (Coulter Immunology, Hialeah, Florida). Direct panning was performed by coating polystyrene Petri dishes with anti-Mo2 diluted in 0,05 M Tris for 1 hour, followed by blocking remaining uncoated plastic with 1% bovine serum albumin. The mononuclear preparation was incubated in these dishes and monocytes were specifically bound by the antibody. However, the yield obtained was poor and it was not practical to increase the amount of coating anti-Mo2, so an indirect method was investigated. This involved coating polystyrene Petri dishes with anti-mouse IgM antibody (Sigma, St. Louis, Missouri), 10 /ug/ml, and blocking with bovine serum albumin. The mononuclear cells were incubated at 37°C for 30 minutes with various dilutions of anti-Mo2 antibody and then washed twice in PBS. The cell suspension was then added to the coated Petri dishes which were incubated at 37°C for 1 hour and monocytes with surface anti-Mo2 were bound by the anti-mouse IgM. Unbound cells were removed by washing gently with PBS.

2.2.2 Assessment of monocyte separation techniques

The yield of mononuclear cells obtained by Ficoll-hypaque centrifugation was assessed by counting methyl green-stained cells in a haemocytometer. Dilution of blood with saline was recommended by Boyum (1968) and control experiments confirmed that this improved the yield approximately 25% compared with undiluted blood. 100 ml of blood yielded 0,7 to 2,8 x 10^8 mononuclear cells, being 52 to 100% of the total mononuclear count calculated from an automated white cell

count and differential count (H1 counter, Technicon, Terrytown, New York). The yield of monocytes obtained after adhesion and panning was assessed by counting the adherent cells after removal with a rubber policeman or, for the foetal calf serum coated plates, release of adherent cells with 0,2% EDTA. Purity of the monocyte preparation was demonstrated by means of phagocytosis of Candida sp. (Miller, 1969) and by non-specific esterase staining (Yam et al, 1971). Candida was cultured in brain-heart infusion broth and heat inactivated at 100°C for 30 minutes. After washing in PBS, 0,5 ml yeast suspension (10^9 organisms per ml) was incubated with 0,5 ml autologous serum, and the yeast was then added to the Petri dishes containing the monocytes, in a ratio of approximately 100 yeast organisms per monocyte. After incubation at 37°C for 1 hour the plates were washed with PBS and the cells which ingested yeast were counted.

The yield obtained by adhesion (81 ± 6,9% of the calculated number of monocytes) was superior to that obtained using foetal calf serum coated dishes (38,2 ± 19,5%) or indirect panning using the highest concentration of Mo2 antibody (21,4 ± 10,2%). More than half (55-72%) of the adherent cells phagocytosed yeast particles and greater than 90% were positive for non-specific esterase. Since a high yield of monocytes was required in order to obtain sufficient RNA for probing, the adhesion method was subsequently used.

2.3 RIBONUCLEIC ACID PREPARATION

2.3.1 RNA extraction

RNA preparation is frequently complicated by degradation of the nucleic acid by ubiquitous RNAases. These small molecular weight

enzymes are active over a wide range of pH and are highly resistant to heating (Blumberg, 1987). A number of precautions were therefore necessary: (i) glassware was baked at 250°C for 4 hours prior to use; (ii) only new disposable plasticware was used, after autoclaving at 120°C for 30 minutes; (iii) solutions were made up of high quality or RNAase free chemicals and prepared with water treated with diethylpyrocarbonate (DEPC), an RNAase inhibitor, at a concentration of 1 ml per litre for 12 hours prior to autoclaving; (iv) gloves were worn at all stages to prevent contamination by RNAases.

RNA was extracted from monocytes using the method described by Chomczynski and Sacchi (1987). The denaturing solution (guanidinium isothiocyanate 4 M; sodium citrate 25 mM; sarcosyl 0,5% [w/v]; 2-mercaptoethanol 0,1 M) was added directly to the Petri dishes containing adherent monocytes. 1 ml was added to a Petri dish and after scraping with a rubber policeman was transferred to a second Petri dish and thereafter to a 2,2 ml Eppendorf reaction tube. For extraction from hepatic and splenic tissue, approximately 100 mg of tissue was added to 1 ml denaturing solution and immediately homogenized with an Ultra-Turrax homogenizer. The resulting viscous mixture was phenol extracted by adding to each 1 ml of solution, 100 /ul sodium acetate (4 M, pH4), 1 ml DEPC-water saturated phenol and 200 /ul chloroform-isoamyl alcohol (49:1). This was cooled on ice for 15 minutes and centrifuged at 10 000 g for 20 minutes at 4°C. The upper aqueous phase was transferred to a new tube and RNA was precipitated by addition of an equal volume of isopropanol and incubation on dry ice for 10 minutes. The RNA was pelleted by centrifugation at 10 000 g for 20 minutes, redissolved in 300 /ul of denaturing solution and reprecipitated with isopropanol. The

resulting RNA pellet was washed in 75% (v/v) ethanol, vacuum dried and dissolved in 20 to 50 /ul DEPC-water. Specimens were stored as an aqueous solution frozen at -70°C.

The RNA obtained was assessed for quantity, purity and degradation by spectrophotometry and electrophoresis. The spectrophotometric scan (figure 1) demonstrated a peak at 260 nm and the absorbance at 260 nm was used to calculate the yield of RNA based on an absorbance of 1 being equivalent to 40 /ug/ml (Davis, 1986). The shape of the scan and an A_{260}/A_{280} ratio of 1,75 to 2,0 confirmed the absence of significant contaminating protein. All samples were electrophoresed in a formaldehyde agarose gel to monitor integrity of the nucleic acid.

2.3.2 RNA electrophoresis

RNA was electrophoresed in a denaturing formaldehyde agarose minigel stained with ethidium bromide (Maniatis et al, 1982). RNA solution (4,5 /ul containing 1 - 5 /ug RNA) was added to 14,5 /ul sample buffer (deionized formamide 10 /ul; formaldehyde 3,5 /ul; running buffer 1 /ul) and denatured for 15 minutes at 55°C. Loading buffer 2 /ul (glycerol 50% [w/v]; EDTA 1 mM; bromophenol blue 0,4% [w/v]; xylene cyanol 0,4% [w/v]) and ethidium bromide (1 mg/ml) 1 /ul were added. The RNA was electrophoresed in a 1,5% agarose minigel containing 2,2 M formaldehyde using a MOPS running buffer (morpholinopropanesulfonic acid 0,2M pH 7,0; sodium acetate 50 mM; EDTA 1 mM). After 2 to 3 hours the gel was viewed on an ultraviolet light source and the presence of the 28S and 18S ribosomal RNA bands confirmed the isolation of undegraded RNA (figure 2).

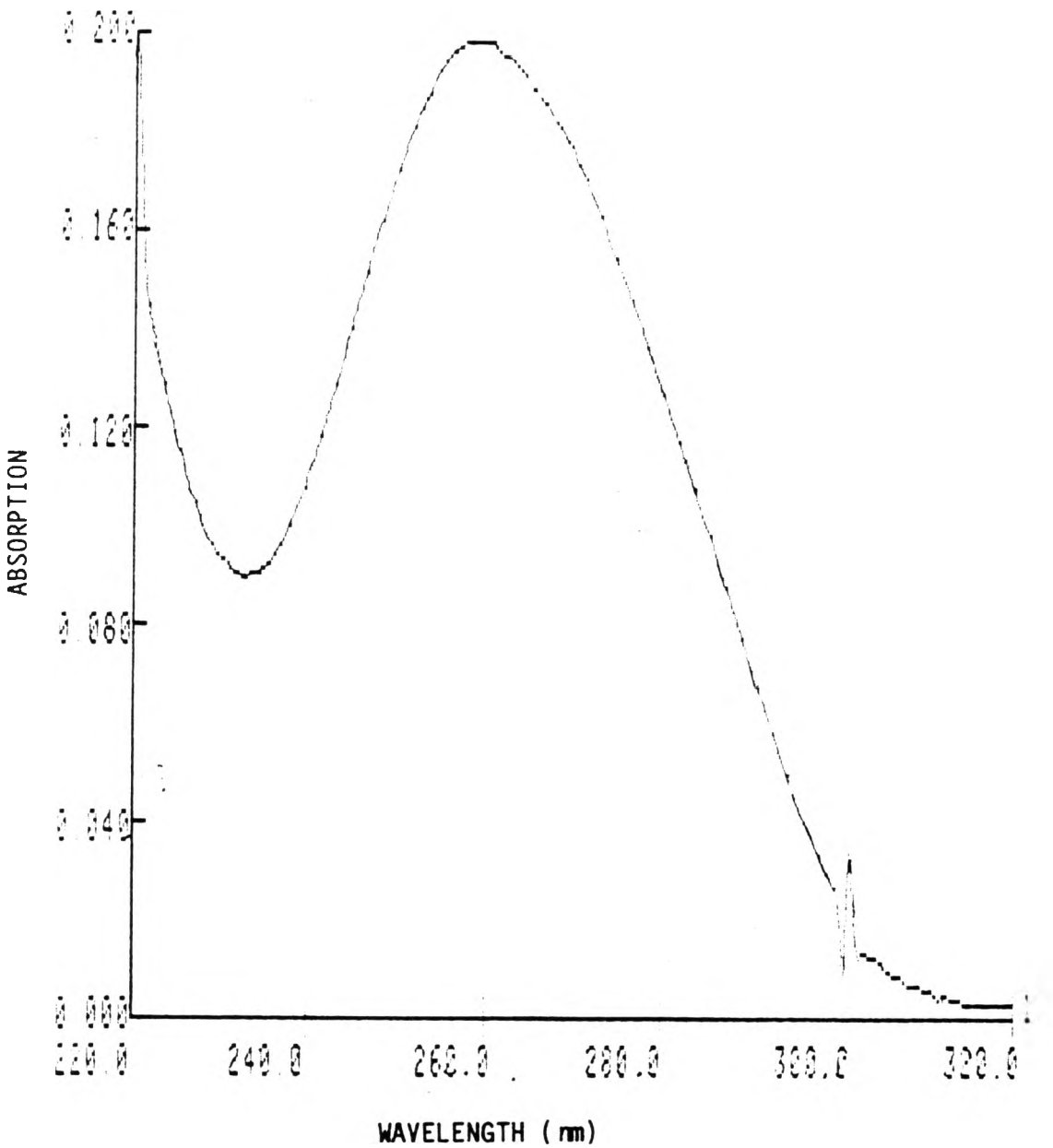


Figure 1: Spectrophotometric scan from 220 to 320 nm of a specimen of extracted RNA

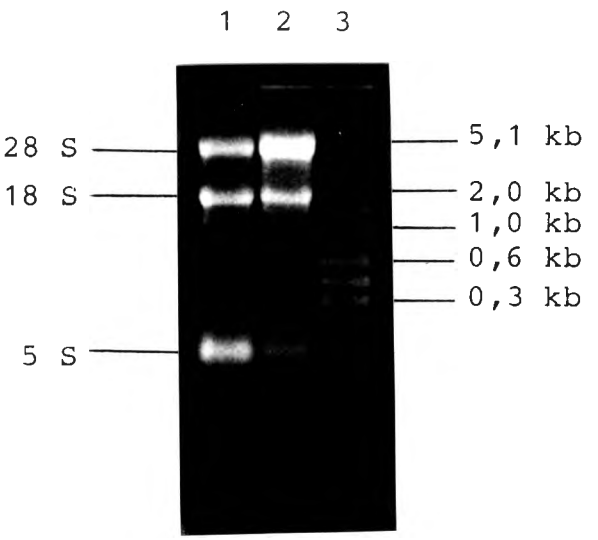


Figure 2: Ethidium bromide stained agarose gel electrophoresis of human monocyte total RNA (lane 1); rat liver total RNA (lane 2); and RNA size markers (lane 3).

2.4 RIBONUCLEIC ACID TRANSFER

RNA was transferred to nylon membrane either as a dot-blot, or after electrophoresis as a northern blot (Meinkoth and Wahl, 1984), prior to hybridizing with radiolabelled probes.

Northern transfer onto Hybond N nylon membrane (Amersham, UK) was performed as recommended by the manufacturers. After electrophoresis of RNA in formaldehyde agarose, the gel was washed twice in 10x SSC (NaCl 1,5 M; sodium citrate 0,15 M) for 20 minutes and then inverted onto a glass plate. Hybond N membrane was placed directly onto the gel, with 2 pieces of Whatman 3 MM paper and a 5 centimetre stack of absorbent pads above, compressed with a glass plate and a 1 kilogram weight. Transfer was allowed to proceed for 6 to 12 hours. The nylon membrane was then air dried and exposed to ultraviolet light for 5 minutes to covalently bond the nucleic acid to the membrane. Blots were stored in cling wrap at 4°C.

Dot-blots and slot-blots on Hybond N membrane were performed using a Minifold dot or slot-blot apparatus (Schleiecher and Schuell, Keene, New Hampshire). RNA (2 to 5 /ug) in 50/ul 20x SSC (NaCl 3 M; sodium citrate 0,3 M) was added to 50 /ul deionized formamide and denatured at 55°C for 15 minutes. This was placed in a well and drawn through the membrane by suction, then washed with 100 /ul 20x SSC. The nylon membrane was air dried, U-V exposed and stored at 4°C.

2.5 cDNA PROBES

Complementary DNA (cDNA) is double stranded DNA made from the corresponding mRNA using reverse transcriptase (RNA-dependant DNA polymerase). It differs from genomic DNA in that it lacks introns (intervening sequences), which are spliced out during formation of

the mRNA. It therefore consists of coding sequences flanked by the 5' and 3' non-coding regions. Full length cDNA transcripts for a number of proteins have been isolated from species-specific cDNA libraries, and cloned into suitable plasmid vectors. The following cDNA probes were used in this study to identify the presence of specific mRNAs:

2.5.1 Human H-ferritin

This clone, pHF 16, was obtained from Dr. J. Drysdale, Tufts University School of Medicine, Boston, MA. The full length transcipt had been isolated from a human liver cDNA library by screening with a previously isolated H-subunit cDNA clone (Boyd et al, 1984 & 1985). The cDNA insert consists of a 77 base pair 5' non-coding region, a 552 base coding and a 161 base 3' non-coding region ending in a poladenylate tail. It is situated in the Pst I restriction site of the plasmid vector pKT 218, a pBR 322 derivative, with a 530 base deletion (Talmadge et al, 1980). The restriction map (figure 3), based on Talmadge (1980) and Boyd (1985) was confirmed by agarose gel electrophoresis after digestion with enzymes Pst I, Rsa I and Eco RI. The fragments used for probing RNA blots were the 430 base Pst 1-Rsa 1 fragment (figure 5), or the 496 and 308 base Pst 1 fragments. The plasmid was obtained in E. coli culture, and was maintained by tetracycline selection.

2.5.2 Human L-ferritin

Clone pLF 108 was also obtained from Dr. J. Drysdale and is a full length cDNA isolated from a liver library by screening with an oligonucleotide probe (Boyd et al, 1985). It consists of a 151 base 5' non-coding region, a 143 base 3' non-coding region and a 528 base

reading frame. The insert is also in the Pst 1 restriction site of pKT 218, leaving the tetracycline resistance gene intact. The fragments used for probing were the 228 base, 346 base and 237 base Pst 1-Hind III fragments (figure 5). The restriction map is shown in figure 4.

2.5.3 Human β -actin

This cDNA, pHF β -A1, was obtained from Dr. I. Parker, University of Cape Town with permission from Dr. L. Kedes, Veterans Administration Medical Centre, Palo Alto, California. It contains an approximately full length cDNA including a poly-A tail, for human fibroblast β -actin in the Okayama-Berg cloning expression vector (Gunning et al, 1983). The clone is maintained by ampicillin selection and the insert is released by digestion with Bam H1 as a 2,0 kb fragment (figure 5). Although a human β -actin cDNA, this clone will hybridize to actin sequences from all higher eukaryotes.

2.5.4 Rat ferritin subunits

These clones were obtained from Dr. H. Munro, MIT, Boston, Massachusetts. The rat L-ferritin subunit, clone pRLFL, was isolated from a rat liver cDNA library by screening using a labelled anti-rat ferritin antibody after hybrid release translation (Brown et al, 1983). The insert is 830 bases in length, being approximately 75% of the total mRNA, and is cloned into the Pst 1 site of pBR 322. The clone is maintained by selection with tetracycline, and is released by digestion with Pst 1, there being no internal Pst 1 sites (figure 6).

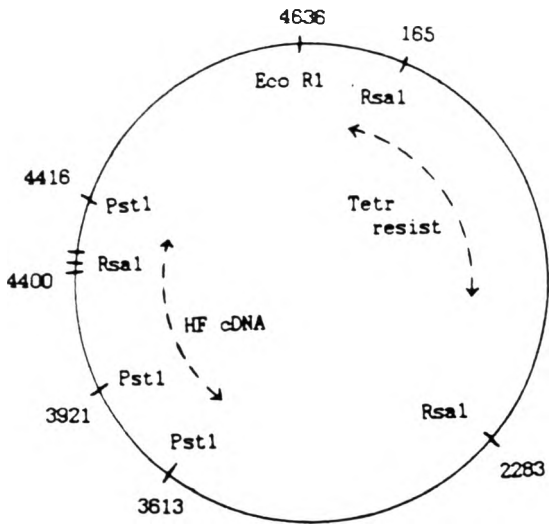


Figure 3: Partial restriction map of the human H-ferritin cDNA containing plasmid, pHF 16.

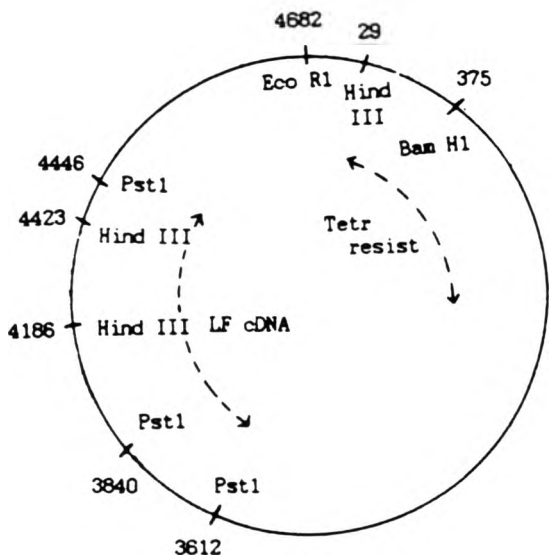


Figure 4: Partial restriction map of the human L-ferritin cDNA containing plasmid, pLF 108.

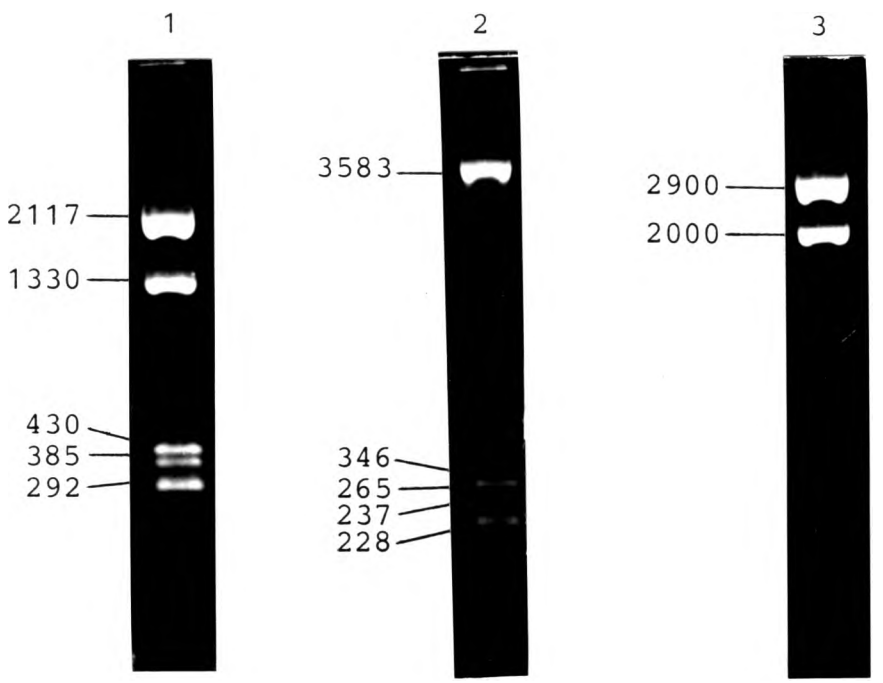


Figure 5: Ethidium bromide stained agarose gel electrophoresis of DNA fragments after restriction digestion of pHF 16 with Pst 1 and Rsa 1 (lane 1), pLF 108 with Pst 1 and Hind III (lane 2), and pHF β-A1 with Bam H1 (lane 3).

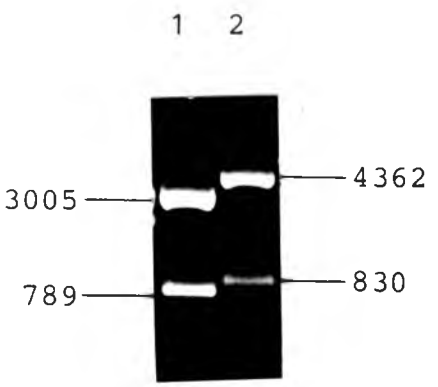


Figure 6: Ethidium stained agarose gel electrophoresis of DNA fragments of the rat H-ferritin cDNA-containing plasmid digested with Pst 1 (lane 1) and L-ferritin cDNA-containing plasmid digested with Eco R1 (lane 2).

The rat H-ferritin subunit clone consists of a 789 base cDNA insert situated in the Eco Rl site of the cloning expression vector SP 65 (Promega). This vector contains the ampicillin antibiotic resistance gene, and the insert is released whole by digestion with Eco Rl (figure 6). These probes were obtained as lyophilized plasmid preparations and were transformed into E. coli HB101.

2.6 PLASMID PREPARATION

2.6.1 Introduction

In order that sufficient rat ferritin cDNA-containing plasmid would be available for multiple hybridizations, these plasmids were transformed into E. coli HB101.

The human ferritin subunit and β -actin cDNA-containing plasmids were obtained in E. coli cultures. These were grown in culture broth with selection and the plasmids were extracted by alkaline lysis (Maniatis et al, 1982). Two different methods were used for plasmid purification, namely caesium chloride density gradient centrifugation and polyethylene glycol precipitation.

2.6.2 Transformation

This was performed according to the method of Chung and Miller (1988). E. coli HB101 was grown in 50 ml Luria-Bertani medium (LB medium: tryptone 1% [w/v]; yeast extract 0,5% [w/v]; NaCl 1% [w/v]; pH 7,5) to early exponential phase (OD_{600} = 0,5). The bacteria were recovered by centrifugation at 5000 g for 10 minutes at 4°C, and resuspended in 5ml of transformation and storage buffer (TSB: LB medium, pH 6,1 with 10% [w/v] PEG MW 3350; 5% [v/v] DMSO; 10mM $MgCl_2$;

10mM $MgSO_4$). After incubation on ice for 10 minutes, a 0,1 ml aliquot was used for transformation. Plasmid DNA (1 ng) was added to the 0,1 ml aliquot, and incubated on ice for 30 minutes. In order to allow expression of the antibiotic resistance gene, 0,9 ml of TSB with 20mM glucose was added and this was incubated at 37°C for 1 hour with vigorous shaking. The culture (100 /ul) was then plated on appropriate antibiotic agar (tetracycline for rat L-ferritin, ampicillin for rat H-ferritin). Approximately 20 transformed clones were obtained on each plate. Miniplasmid preparations were performed on a number of these clones by a scaled down version of the alkaline lysis and PEG precipitation method described below (section 2.6.3 and 2.6.4). Extracted plasmids were then digested with restriction endonucleases (see section 2.7.1), to linearize the plasmid (Bam Hl), and to release the cDNA insert (Pst 1 for the rat L-ferritin cDNA and Eco Rl for the rat H-ferritin cDNA). Electrophoresis in agarose gels with ethidium bromide staining confirmed the presence of the plasmid and insert in each transformed colony. The clones were grown up in Luria-Bertani medium with antibiotic selection, and stored in 25% (w/v) glycerol at -70°C.

2.6.3 Plasmid isolation

This was performed essentially as described by Maniatis and colleagues (1982). 500ml of Luria-Bertani medium with the appropriate antibiotic (ampicillin 50 /ug/ml or tetracycline 12,5 /ug/ml) was inoculated with the plasmid-containing E. coli culture. This was incubated at 37°C overnight and the bacteria were harvested by centrifugation at 5000 g for 10 minutes and washed in a solution of Tris-HCl (10mM, pH 7), EDTA (1mM) and NaCl (100mM). After

centrifugation, the bacteria were resuspended in 10 ml ice-cold TGE (Tris-HCl pH8 25 mM; glucose 50 mM; EDTA 10 mM). Lysozyme (60 mg) was added, dissolved by shaking and the solution allowed to stand at room temperature for 10 minutes. Freshly prepared NS solution (NaOH 200 mM, SDS 1% [w/v]), 20 ml, was added and the solution was put on ice for 10 minutes. Ice cold potassium acetate (potassium 3 M, acetate 5 M), 15 ml, was added followed by incubation on ice for a further 10 minutes. Centrifugation at 76 000 g for 20 minutes at 4°C pelleted the cell debris and chromosomal DNA. The supernatant, containing predominantly plasmid DNA and bacterial RNA was purified using one of the following two methods.

2.6.4 Plasmid purification

Plasmid DNA was purified by isopycnic centrifugation in a caesium chloride-ethidium bromide density gradient (Maniatis et al, 1982). This method depends on the buoyant density of the DNA in the presence of ethidium bromide, a dye which binds to DNA causing unwinding of the molecule. While this tends to decrease the density of the DNA, closed circular plasmid DNA is supercoiled and retains a higher density than other types of DNA (Wilson and Goulding, 1986). Ultracentrifugation of the caesium chloride (CsCl) solution causes sedimentation of the CsCl molecules, to form a density gradient. The DNA molecules will either rise or sediment to form a band at their isopycnic position.

DNA was precipitated from the supernatant resulting from the alkaline lysis procedure by adding 0,6 volumes of isopropanol and allowing to stand at room temperature for 15 minutes. This was pelleted at 12 000 g for 30 minutes at room temperature. The pellet

was washed in 75% (v/v) ethanol, vacuum dried, and dissolved in 4 ml TE buffer (Tris HCl pH 8 10mM, EDTA 1mM). Caesium chloride solution (1g/ml) was added to give a density of 1,55 g/ml. The correct density of the CsCl solution was confirmed by either weighing 1 ml of the solution or by measuring the refractive index. Ethidium bromide was added to a final concentration of 0,8 mg/ml, the solution was sealed in a Beckman centrifuge tube and centrifuged in a Beckman TL 100 ultracentrifuge in a 100.3 angle rotor at 65 000 rpm (158 000 g at r_{av}) for 24 hours at 20°C. The closed circular plasmid DNA formed a band in the mid-zone of the gradient, below a band of nicked plasmid and chromosomal DNA, while RNA pelleted to the bottom. The plasmid band was harvested by piercing the tube with an 18 G needle and ethidium bromide removed by 1-butanol extraction. TE buffer, 2,7 volumes, was added to correct the solute concentration and the DNA precipitated with isopropanol. After ethanol washing and drying, the DNA was dissolved in TE buffer and quantified spectrophotometrically.

A second method of plasmid purification made use of the ability of polyethylene glycol (PEG) to precipitate plasmid DNA without precipitating the ribonucleic acid. The supernatant resulting from the alkaline lysis extraction was treated with RNAase A at a final concentration of 20/ug/ml at 37°C for 20 minutes. The solution was extracted with an equal volume of phenol-chloroform (phenol with 0,1% 8-hydroxy-quinoline equilibrated with 0,1 M Tris pH8 and chloroform:isoamyl alcohol 24:1) and centrifuged at 5 000 g for 10 minutes to separate the phases. The upper aqueous phase was harvested and the solute concentration was corrected by addition of 1/10 volume of sodium acetate (3 M, pH 7). DNA was precipitated with 0,6 volumes isopropanol at -70°C and the precipitate was pelleted, washed in

ethanol and dried. This nucleic acid pellet was dissolved in 1,6 ml water, the solute concentration adjusted by addition of 0,4ml NaCl (4 M), and then 2ml PEG (13% [w/v], MW 8000) was added. After incubation on ice for 10 minutes this was centrifuged at 10 000 g and the pellet was washed, dried and redissolved in TE buffer. Plasmid DNA was quantified spectrophotometrically.

2.7 PREPARATION OF LABELLED PROBE

2.7.1 Restriction Digestion

Digestion of plasmid DNA with restriction endonucleases was used to confirm the presence and identitiy of the plasmids, and to release the cDNA inserts. Restriction enzymes were obtained from Boehringer Mannheim (Mannheim, West Germany) and their incubation buffers used. The restriction maps of the various plasmids are discussed in section 2.5 above. The following enzymes were used to release the cDNA inserts:

Plasmid	Enzyme	Fragment used	Buffer
pHF 16	Pst 1/Rsa 1	427 base	L(+ NaCl)
pLF 108	Pst 1/Hind III	346,237,228 base	M(+ $^1/_2$B)
pHF A-1	Bam Hl	2,0 kb	B
pRLFL	Pst 1	830 base	H
Rat HF	Eco Rl	790 base	H

Restriction digestion was carried out in a 20/ul volume. Water was added to plasmid DNA (2 to 10/ug) to a volume of 18/ul, followed by 2/ul of the appropriate buffer and 10 to 20 units of the restriction

endonuclease. The double digestion of pHF 16 was performed using first Rsa 1 in buffer L and then Pst 1 after addition of 1/ul NaCl (2 M). pLF 108 was digested first with Hind III in buffer M, followed by Pst 1 after adding 1/ul of buffer B to approximate the high ionic strength required by the latter enzyme. Incubation was at 37°C for 1 hour after which the reaction was stopped by addition of 0,5/ul EDTA (0,5 M). Fragments were denatured at 65°C for 5 minutes then rapidly cooled on ice. Loading buffer, 2/ul, (glycerol 50% [w/v], EDTA 1mM, bromophenol blue 0,4% [w/v], xylene cyanol 0,4% [w/v]) was added and the plasmid DNA electrophoresed in a 1,2% or 1,5% agarose minigel. The lower concentration was used to resolve fragments bigger than 0,5 kb and the higher concentration was used for smaller fragments. Ethidium bromide at a concentration of 0,5/ug/ml was added to both the gel and the running buffer. Electrophoresis was performed in TBE buffer (0,089M Tris-HCl pH 8; 0,089M boric acid; 2 mM EDTA) at 50 volts for approximately 3 hours after which the gel was viewed on an ultraviolet source. Figures 5 and 6 demonstrate the electrophoresis of the 5 plasmids after restriction digestion to release their inserts.

For preparative gels, low gel temperarure agarose (Bio-Rad, Richmond, California) was used, with electrophoresis being performed at 4°C. While radiolabelling of cDNA in agarose is possible, difficulties were encountered due to coagulation of the solution when placed on ice and a difficulty in obtaining a high enough cDNA concentration for the radiolabelling procedure. The cDNA was subsequently purified from the agarose as follows. The appropriate band was cut out of the agarose and was dissolved in about 1 ml distilled water for 20 minutes at 65°C. NaCl was added to a final

concentration of 100 mM and the solution was extracted with an equal volume of phenol in TE buffer (prewarmed to 37°C), followed by extraction with chloroform:isoamyl alcohol (24:1). The aqueous phase was precipitated with 0,6 volume isopropanol and the pellet was dissolved in 50 to 100 /ul TE buffer. To check the concentration of the DNA, a 2/ul sample was spotted on cling wrap on a U-V source with 2/ul each of control DNA with a concentration of 1 to 100 ng/ml. 2 /ul of TE containing ethidium bromide (2/ul/ml) was added to each spot and the brightness of the fluorescence of the sample was compared with the controls (Maniatis et al, 1982).

2.7.2 Random Primer Labelling

cDNA fragments were radiolabelled with ^{32}P by the random primer method (Feinberg and Vogelstein, 1983) using a kit obtained from Boehringer Mannheim (Mannheim, West Germany). This method uses a mixture of random hexamers which hybridize to the cDNA. New DNA is synthesized using the Klenow fragment of DNA polymerase with the cDNA acting as a template and the hexamer as a primer. Labelled nucleotides are incorporated in the newly synthesized DNA. cDNA (50 to 200 ng) in a maximum volume of 9/ul was added to a reaction tube, denatured at 100°C for 2 minutes and immediately put on ice. To this was added 3/ul of nucleotide mix (dATP, dGTP, dTTP), followed by 2/ul of reaction buffer with random hexamers, 5/ul of α -^{32}P dCTP (3000 Ci/mmol) (Amersham, UK), and 1/ul Klenow enzyme. This was incubated at 37°C for 30 to 90 minutes. The reaction was monitored by means of assessing the percentage of label incorporated in the synthesized DNA probe. Reaction mix (1 /ul) was added to 200 /ul water containing 1 mg/ml carrier DNA. An aliquot (50 /ul) was placed in a scintillation

vial and another aliquot (50 /ul) was precipitated by adding it to 2 ml 10% (w/v) trichloracetic acid (TCA). This was filtered using a Whatman GFC glass microfibre filter disc and washed thoroughly with 10% TCA. Both the filter and the 50 /ul control were counted in a scintillation counter and the percentage incorporation calculated as follows:

$$\% \text{ incorporation} = \frac{\text{counts on filter}}{\text{counts in solution}} \times 100$$

The amount of synthesized DNA (in ng) in the labelling reaction was determined by the formula:

$$\frac{/\text{uCi dCTP (50/uCi)} \times 13{,}2 \times \% \text{ incorporation}}{\text{specific activity of dCTP (3000 Ci/mmol)}}$$

The specific activity of the labelled probe in dpm//ug was calculated according to the following formula:

$$\frac{/\text{uCi dCTP (50/uCi)} \times 2{,}2\text{x}10^4 \times \% \text{ incorporation} \times 10^3}{\text{input DNA + newly synthesized DNA (ng)}}$$

Using this method, the percentage incorporation of labelled probes was found to range from 31% to 75% with a specific activity of 8,6 x 10^8 to 2,0 x 10^9 dpm//ug. Although it is recommended that for incorporation of less than 50% the unbound label should be removed using a spin column in order to reduce background radioactivity, this was not found to be necessary. Each labelling procedure was checked for specificity and background by northern blotting and it was found that at the high stringency wash used, non-specific background radioactivity was not a problem. Initially suboptimal hybridization results were obtained and this was found to be due to the use of greater than 200 ng cDNA in the labelling reaction.

2.8 HYBRIDIZATION

Northern blots and dot-blots were hybridized with the labelled cDNA probes essentially as described by the manufacturers of Hybond N membrane (Amersham, UK). The membrane was prehybridized in a solution made up of 6,25 ml 20x SSPE (NaCl 3,6 M; phosphate buffer 0,2 M, pH 7,7; EDTA 0,02 M); 12,5 ml deionized formamide; 1,25 ml 100x Denhardts solution (Ficoll 2% [w/v], polvinyl pyrollidone 2% [w/v], bovine serum albumin 2% [w/v]); denatured herring sperm DNA 2 mg; water 4,75 ml; and 1,25 ml 10% (w/v) sodium dodecyl sulphate (SDS). Each blot was placed in 5 to 15 ml of this solution in a heat seal bag and incubated at 42°C for at least 4 hours. This was then replaced with 4 to 10 ml of a hybridizing solution similar to the prehybridizing solution but containing only 0,5 mg herring sperm DNA, no SDS and 4% (w/v) polyethylene glycol (MW 8000). To this was added labelled cDNA probe after denaturing at 100°C for 2 minutes. An aliquot of 1 /ul of the 20 /ul labelling reaction was added to each northern blot and 10 to 16 /ul to each dot-blot (depending on the number of dots) and incubated at 42°C for 12 hours with gentle shaking.

Following hybridization, the blots were washed to a high stringency. The initial wash was in 2x SSC (0,3 M NaCl; 0,03 M sodium citrate) with 0,1% (w/v) SDS at 42°C for 15 minutes. This was followed by a wash in 1x SSC with 0,1% (w/v) SDS for 30 minutes and then a high stringency wash in 0,5x SSC with 0,1% SDS at 65°C for 15 minutes. This high temperature, low ionic strength wash would cause dissociation of all but specifically bound probe. The high stringency wash was omitted when rat RNA was probed with the human β -actin probe.

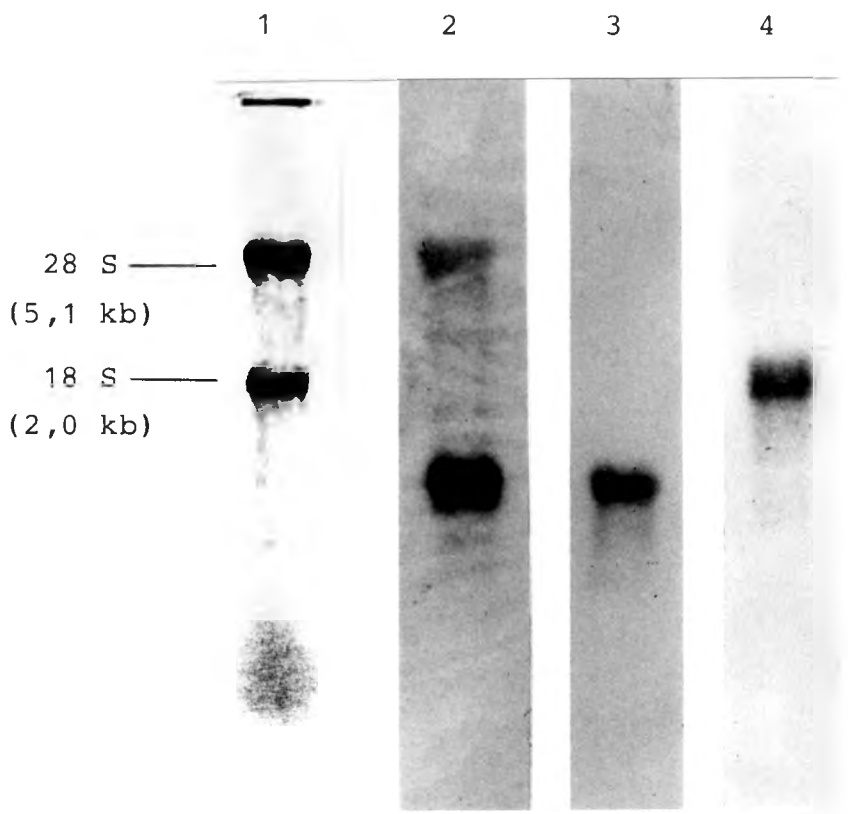


Figure 7: Northern blot of human monocyte total RNA stained with methylene blue (lane 1); and autoradiographs of northern blots probed for the human H-ferritin subunit (lane 2), L-ferritin subunit (lane 3) and β-actin (lane 4).

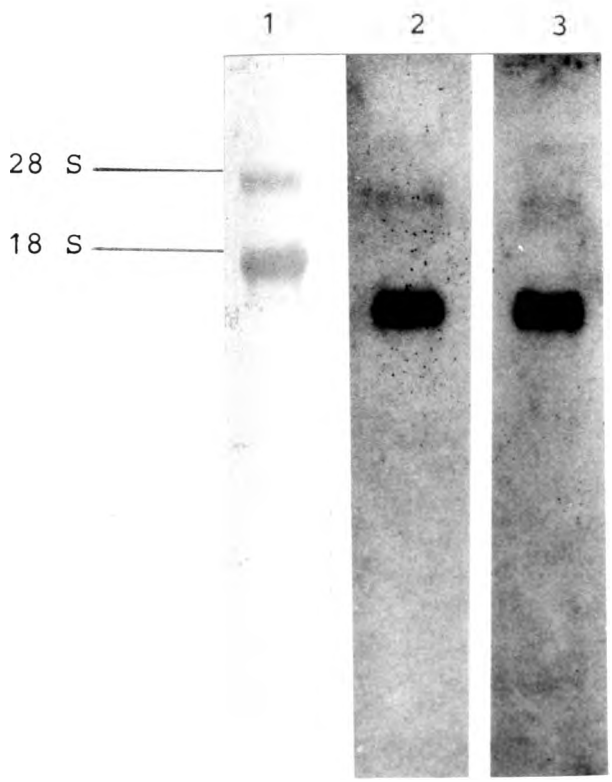


Figure 8: Northern blot of rat liver total RNA stained with methylene blue (lane 1), and probed for rat H-ferritin subunit (lane 2) and rat L-ferritin subunit (lane 3).

Blots were air dried and then wrapped in cling wrap prior to autoradiography. Kodak XAR 5 film was exposed with intensifying screens at -70°C for 4 to 36 hours and developed in an automated X-ray film developer. The autoradiographed dots were assessed visually and were quantitated by scintillation counting of the dots cut from the membrane. Figures 7 demonstrates northern blots of human monocyte RNA probed with the human ferritin and β-actin probes and figure 8 shows rat spleen RNA probed with the rat ferritin subunit probes.

2.9 RNA FRACTIONATION

2.9.1 RNAase inhibitors

RNA was fractionated by density gradient centrifugation into free cytoplasmic mRNA and ribosome-bound mRNA in order to investigate changes in the distribution of ferritin mRNA associated with inflammation. As guanidinium isothiocyanate would disrupt the ribosomes, this agent could not be used prior to centrifugation. Guanidinium acts as a powerful RNAase inhibitor, an essential requirement for the isolation of intact RNA from animal cells, and a number of other RNAase inhibitors were therefore investigated.

Commercially available human placental RNAase inhibitor (Amersham, UK) was used in concentrations from 50 U/ml to 1000 U/ml as described by Aziz and Munro (1986). Control experiments revealed that marked degradation of splenic RNA occurred, with only slight protection at the highest concentrations (figure 9). Heparin, an RNAase inhibitor (Palmiter, 1973) which prevents protein-nucleic acid interactions, was used in concentrations from 100 to 1000 /ug/ml. Degradation, although less marked, still occurred even when heparin was used in

combination with placental RNAase inhibitor (figure 10). Emtage et al (1974) discussed the use of various RNAase inhibitors and found that heparin and placental RNAase inhibitor had limited spectra of activity, being more active in some tissues than others. Use of higher concentrations of heparin was complicated by the co-precipitation of heparin with the nucleic acid. The heparin could be removed by washing the pellet with a solution of sodium acetate (3 M), EDTA (5 mM), pH 6, which dissolves heparin and DNA but not RNA (Palmiter, 1973). However, control experiments using RNA markers demonstrated that a significant amount of low molecular weight RNA was lost by this procedure.

Ribonucleoside-vanadyl complexes have been used effectively to protect RNA extracted from lymphocytes from degradation (Berger and Birkenmeier, 1979). This agent has a wide spectrum of activity, inhibiting all RNAases, except that from Bacillus cereus, in addition to a number of other ribonucleases and restriction endonucleases (Puskas et al, 1982). Ribonucleoside-vanadyl complexes were made essentially as described by Berger and Birkenmeier (1979). Uridine 2',3'-cyclic monophosphate (2 mmol) was dissolved in 8 ml DEPC treated water in a boiling water bath. While the solution was flushed with nitrogen gas, 1 ml $VOSO_4$ (2 M) was added, the pH was adjusted to 6 with 10 N NaOH and then to 7 with 1 N NaOH. A colour change from blue to green-black was noted, indicating formation of the complexes. The volume was made up to 10 ml and this 200 mM solution was divided into aliquots and stored at -20°C. Addition of this solution to the homogenizing buffer, in combination with placental RNAase inhibitor and heparin in the sucrose gradient, significantly decreased the RNA degradation as assessed by ethidium stained agarose gel electro-

phoresis (figure 11). This method was used in the subsequent RNA fractionation experiments. Some RNA degradation was still noted to occur, as determined by northern blots of the fractionated RNA.

2.9.2 Sucrose density gradient centrifugation

Splenic tissue (50 to 100 mg) was placed in 2 ml ice-cold homogenizing buffer (KCl 0,25 M; $MgCl_2$ 0,01 M; Hepes 0,02 M, pH 7,5; dithiothreitol 2 mM; cyclohexamide 150 /ug/ml; RNAase inhibitor 100 U/ml; heparin 150 /ug/ml; ribonucleoside-vandyl complex 20 mM) and homogenized in an Ultra-Turrax homogenizer (Janke and Kunkel, West Germany). Nonidet P-40 (Sigma, St. Louis, USA) was added to 0,5% concentration and the solution was incubated on ice for 10 minutes. Mitochondria and nuclei were pelleted at 10 000 g for 10 minutes and the post-mitochondrial supernatant was layered onto 14 ml of a 10 to 50% (w/v) sucrose gradient. This was made up with RNAase free sucrose in KCl 0,25 M; $MgCl_2$ 0,01 M; Hepes 0,02 M; dithiothreitol 2 mM; cyclohexamide 150 /ug/ml; RNAase inhibitor 50 U/ml; heparin 150 /ug/ml. After centrifugation for 4 hours at 4°C in a Beckman SW 28 rotor at 26 000 rpm, the gradient was separated into eight 2 ml fractions. RNA was isopropanol precipitated and the pellet was dissolved in guanidinium isothiocyanate. After phenol-chloroform extraction, the RNA was reprecipitated and stored as an aqueous solution at -70°C. Agarose gel electrophoresis of RNA from the 8 fractions demonstrated ribosomal RNA bands in fractions 3, 4 and 5 for splenic RNA and fractions 3 to 7 for liver RNA (figure 11). A trace of messenger RNA was seen in fractions 7 and 8. Agarose gels were northern blotted onto Hybond N nylon membrane for hybridization with labelled cDNA probes.

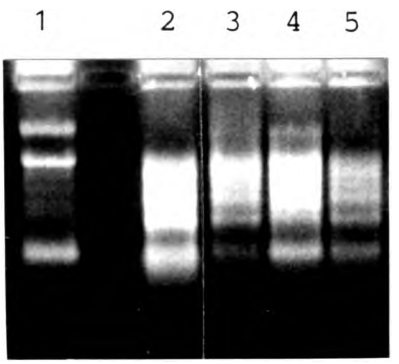


Figure 9: Ethidium stained agarose gel showing control rat spleen RNA (lane 1) and RNA extracted from 100 mg spleen specimens without guanidinium isothiocyanate, using human placental RNAase inhibitor 50 U (lane 2), 200 U (lane 3), 1000 U (lane 4), and 1000 U with a 500 mg specimen (lane 5).

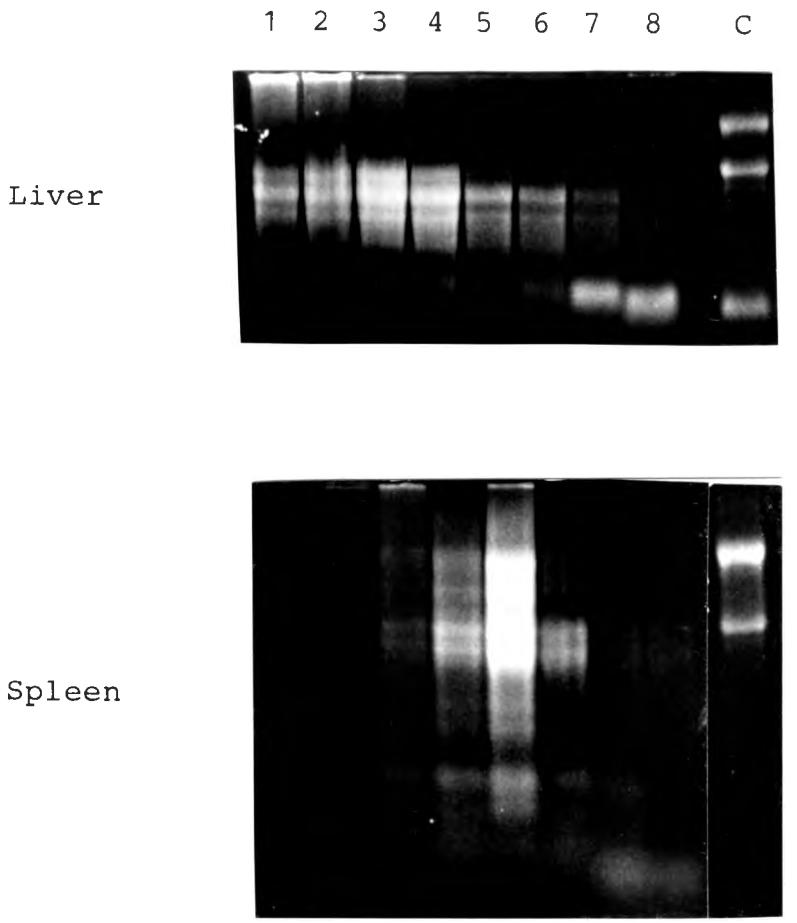


Figure 10: Ethidium stained agarose gels of fractionated liver and spleen RNA, using heparin and human placental ribonuclease inhibitor, demonstrating significant degradation. Lanes 1 to 8 represent fractions harvested from sucrose gradients, lane C is undegraded control RNA.

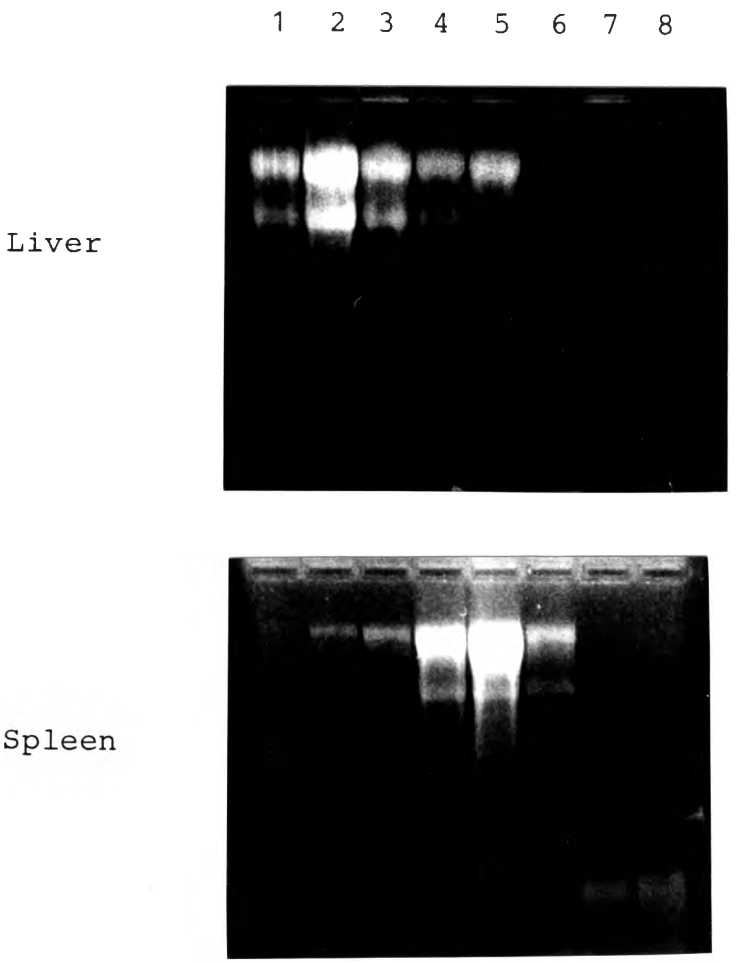


Figure 11: Ethidium stained agarose gels of fractionated rat liver and spleen RNA using vanadyl ribonucleoside complexes as RNAase inhibitor.

3. INVESTIGATION OF FERRITIN mRNA IN INFLAMMATION

3.1 INTRODUCTION

Inflammatory states are known to produce a reduction in the reutilization of iron released from red blood cells (Freireich et al, 1957). This partial block of iron release is associated with an increase in ferritin synthesis (Cartwright and Lee, 1971). The mechanism of this increased reticuloendothelial ferritin synthesis has not been clearly elucidated. Inflammation may have a direct effect stimulating apoferritin synthesis (Konijn and Hershko, 1977), or may cause increased delivery of iron to the reticuloendothelial cell with secondary induction by iron of ferritin production. The latter mechanism would involve the post-transcriptional regulation of ferritin synthesis described by Munro and co-workers (Drysdale and Munro, 1965; Aziz and Munro, 1986), occurring without a rise in cellular ferritin mRNA content. Alternatively, a direct effect of inflammation on ferritin synthesis may occur at a transcriptional or post-transcriptional level. Previous animal studies have demonstrated that the rise in ferritin synthesis occurs prior to the change in serum iron levels, suggesting that ferritin synthesis is the primary event (Konijn and Hershko, 1977). Their later work demonstrated a biphasic rise in ferritin synthesis on induction of inflammation, but their phenotypic expression technique does not adequately allow an assessment of the mechanism of the increased ferritin synthesis (Konijn et al, 1981). The work of Schiaffonati and co-workers (1988) addressed the question of ferritin synthesis in the turpentine treated rat, but investigated only hepatic synthesis of the L-ferritin subunit. No increase in cellular levels of this mRNA was found.

Part I of the present study looked at total cellular content of ferritin subunit mRNA in peripheral blood monocytes of patients with acute and chronic inflammation compared with normal controls, to assess steady state changes in ferritin mRNA concentration. Part II investigated total ferritin subunit mRNA content of hepatic and splenic tissue in rats, at various times after induction of inflammation by turpentine injection. Part III investigated the subcellular distribution of ferritin mRNA in inflamed rats to determine whether redistribution of mRNA from a free pool to polyribosomes occurred.

3.2 PATIENTS WITH INFLAMMATORY DISEASES

3.2.1 Introduction

This study was carried out to find out whether the amount of ferritin subunit mRNA in reticuloendothelial cells was increased in the steady state condition of established inflammation. To this end, ferritin mRNA content of circulating blood monocytes was compared in patients with acute or chronic inflammation and normal controls. The blood monocyte is an accessible circulating reticuloendothelial cell which is known to contain significant concentrations of ferritin, with a rise in concentration on stimulation by iron (Summers et al, 1975). Increased levels of peripheral blood monocyte ferritin have been demonstrated in patients with the anaemia of chronic disease (Summers and Jacobs, 1976) and in acute inflammatory states (Lipschitz et al, 1975).

3.2.2 Subjects and Methods

Approval for the study was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (protocol no. 5/4/88). Adult patients admitted to the Hillbrow and Johannesburg Hospitals with active pulmonary tuberculosis or acute lobar pneumonia were included, after informed consent had been obtained. Pulmonary tuberculosis was diagnosed by the presence of acid fast bacilli on microscopy of sputum stained with Ziehl-Nielsen stain, in association with typical radiographic changes. Lobar pneumonia was diagnosed in patients with a history of acute pyrexial illness and radiographic evidence of lobar pneumonia. Subjects with a haemoglobin concentration of less than 8 g/100ml were excluded. Patients with tuberculosis were entered into the study prior to the institution of

therapy, and those with pneumonia within 18 hours of beginning antibiotic treatment. Control subjects were healthy volunteer staff members from both hospitals.

After informed consent had been obtained, 120 ml blood was obtained for the following tests: (i) a full blood count on an H1 electronic counter (Technicon, Terrytown, New York); (ii) a Westergren ESR; (iii) C-reactive protein concentration by an immunoturbidometric assay (Melamies, 1983); (iv) the serum iron concentration and total iron-binding capacity (International Committee for Standardisation in Haematology, 1978 a&b); (v) the serum ferritin concentration by enzyme linked immunosorbent asssay (Conradie and Mbhele, 1980); (vi) monocyte ferritin mRNA content.

Mononuclear cells were prepared from 100 ml heparinized blood by centrifugation on Ficoll-hypaque (Pharmacia, Uppsala, Sweden) followed by adherence to polystyrene Petri dishes. RNA was extracted from the monocytes by the guanidinium isothiocyanate-phenol-chloroform technique and specimens were stored at -70°C. Equal amounts of total RNA were dot-blotted onto Gene-Screen (New England Nuclear, Boston, MA), and hybridized with cDNA probes. The probes used were the human ferritin H- and L-subunit and human β -actin probes radiolabelled with ^{32}P by the random priming method. Autoradiographs were exposed at -70°C for 6 to 24 hours and dots were quantified by scintillation counting in a liquid scintillation cocktail.

Results of the radioactive counts of the RNA specimens probed with ferritin subunit probes were corrected in terms of the counts obtained with the constitutively expressed protein β -actin.

Student's t test was used to compare the means of counts from the acute inflammation, chronic inflammation and control groups.

3.2.3 Results

Table 1 shows the values for the inflammatory markers and iron indices in control subjects and in patients with tuberculosis and pneumonia. The tuberculosis and pneumonia groups have significantly increased inflammatory marker values, with a mean erythrocyte sedimentation rate of 92 mm/1st hour compared with 4 mm in controls, and a mean C-reactive protein of 119 mg/l compared with a control value of 6,6 mg/l. These inflammatory groups demonstrate a significantly lower haemoglobin concentration, with a decreased serum iron level (mean of 10,8 /umol/l compared with a control value of 15,0 /umol/l), indicative of the reticuloendothelial block to release of iron. A markedly increased mean serum ferritin level of 881/ug/l was found in the inflammatory patients compared with 74/ug/l in the control group. As in previous studies the serum ferritin concentrations were found to follow a skewed distribution (Brink et al, 1976) and accordingly, the results were expressed in terms of the geometric mean and one standard deviation range. These results demonstrate the typical abnormalities of iron metabolism found in patients with inflammation.

Autoradiographs of the dot-blots probed with the ferritin subunit cDNA probes (Figure 12) showed no obvious increase in ferritin subunit mRNA in the inflammatory groups compared with the control group. Quantitative scintillation counting of these dots confirmed that no significant increase had occurred (figure 13). However wide variations in the number of counts occurred between samples in the

inflammatory groups, even when probed with cDNA of the constitutively expressed protein β-actin (figure 13). This can be accounted for by varying increases in other mRNAs synthesised by the activated monocytes of these patients and some variation in the amount of total RNA dotted. To correct for this, the count for each sample hybridized with the ferritin subunit probes was divided by the corresponding count when hybridized with the β -actin probe. The mean and standard error of the mean of these values are depicted graphically in figure 14. No increase was noted in L-ferritin mRNA, and a small, but statistically insignificant decrease ($p > 0,10$) was found when RNA was probed for H-ferritin mRNA.

3.2.4 Conclusions

This study addressed the question of whether the ferritin mRNA content of reticuloendothelial cells was increased in inflammatory diseases in human subjects. No increase was found in patients with an acute (lobar pneumonia) or chronic (pulmonary tuberculosis) infection. While there were wide variations between subjects these were largely eliminated when individual results were corrected for changes in the β-actin control. The validity of this control is confirmed in the following section. Transcriptional regulation of protein synthesis is associated with significant rises in the specific mRNA. The absence of an increase in monocyte ferritin subunit mRNA in the presence of an inflammatory response is compatible with post-transcriptional regulation of ferritin synthesis. This would tend to support the hypothesis that the increased ferritin synthesis in inflammation occurs secondary to a rise in intracellular iron levels.

Table I: Mean (± SD) for inflammatory markers and iron indices in the three groups of subjects (serum ferritin results are given as the geometric mean and SD range)

	Control	Pneumonia	Tuberculosis
n	6	6	6
Age + (years)	43,3 ± 18,7	38,5 ± 12,2	32,5 ± 11,9
Haemoglobin ** (g/dl)	15,6 ± 1,5	13,6 ± 1,4	11,2 ± 1,8
ESR * (mm 1st hour)	4 ± 4	103 ± 34	80 ± 26
C-reactive protein * (mg/l)	6,6 ± 2,3	136 ± 61,8	101 ± 17
Serum Fe * (µmol/l)	15.0 ± 7,8	10,5 ± 2,2	11,0 ± 1,9
Total iron-binding* Capacity (µmol/l)	54,9 ± 6,0	34,8 ± 4,4	32,4 ± 4,5
Percentage + Saturation	26,5 ± 11,5	30,9 ± 9,0	34,1 ± 6,0
Serum ferritin* (µg/l)	74 (15-324)	1096 (451-2667)	708 (316-1585)

* Control group statistically different from pneumonia and tuberculosis groups by t tests with Bonferroni corrections ($P < 0,05$)

** Pneumonia and tuberculosis groups statistically equivalent but different from control group by analysis of variance ($p < 0,0009$)

\+ All groups statistically similar.

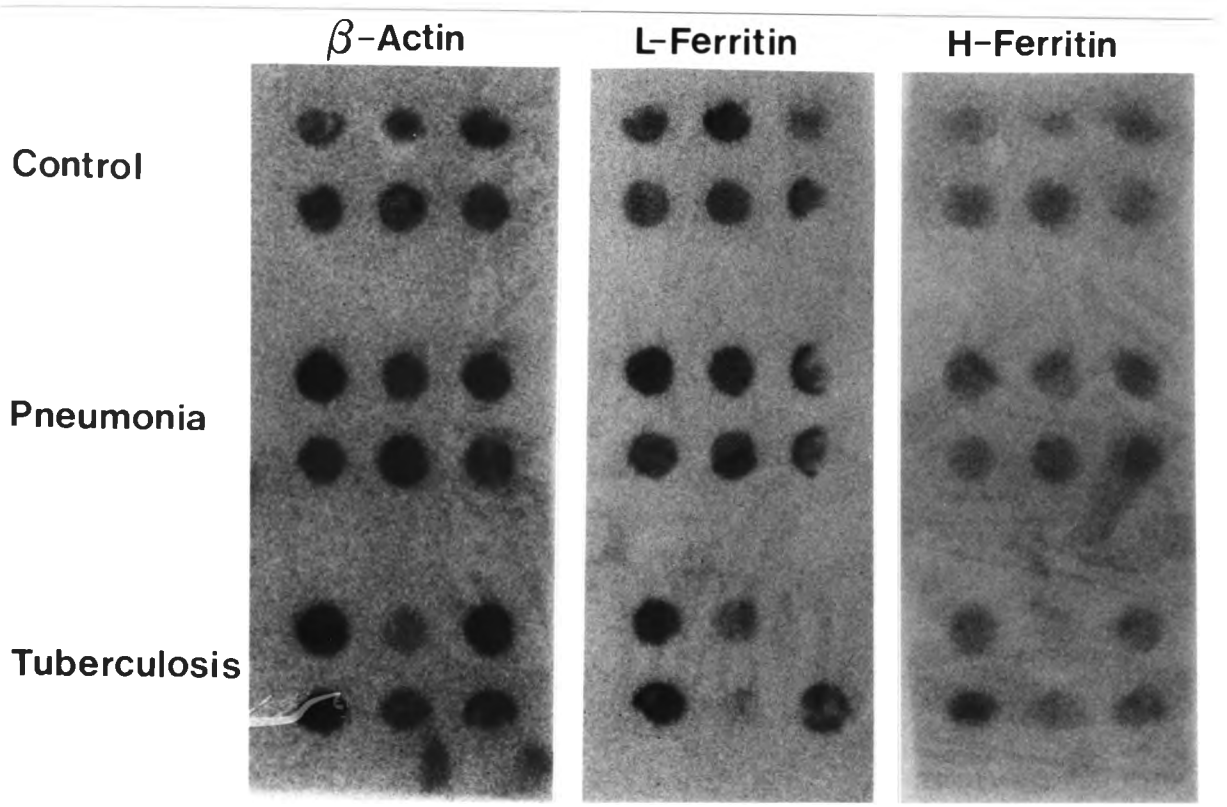


Figure 12: Autoradiographs of dot-blots of RNA specimens from controls and patients with tuberculosis and pneumonia, probed for ferritin subunit and β -actin mRNA.

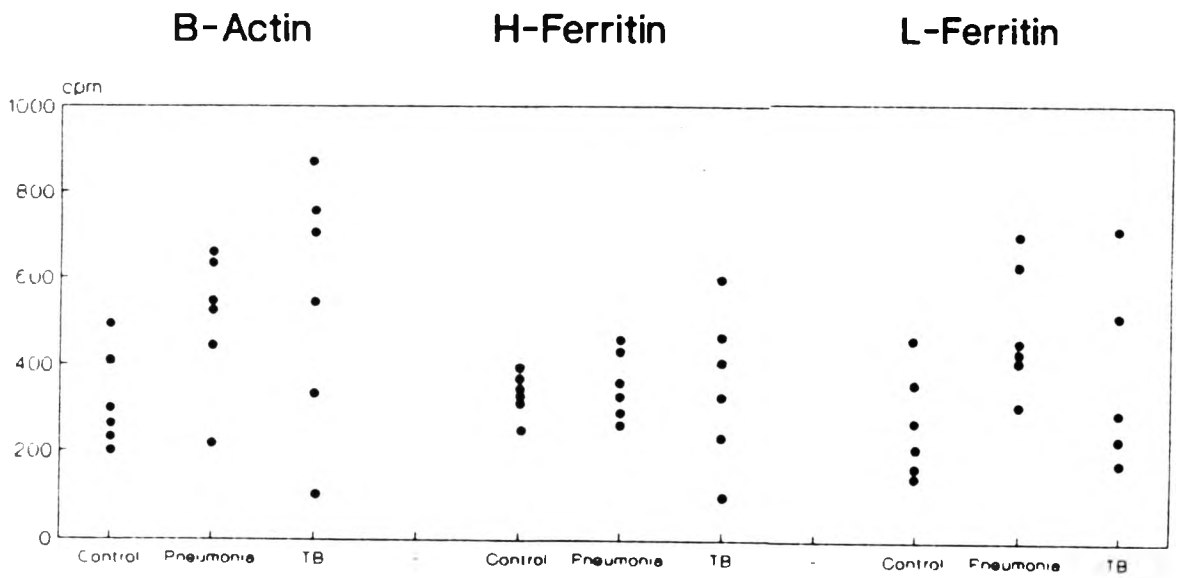


Figure 13: Individual counts of RNA dot-blots probed for ferritin subunit and β -actin mRNA.

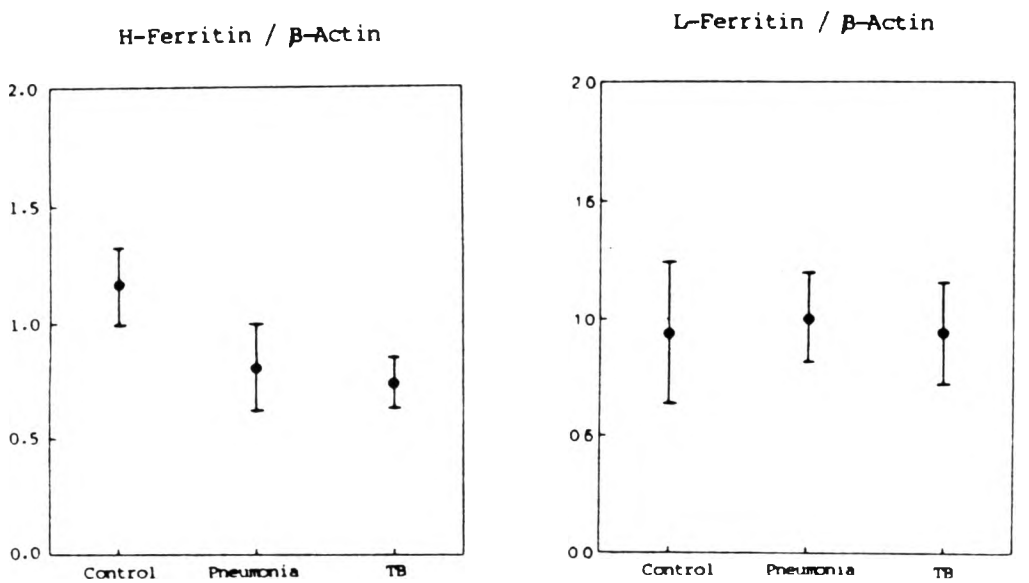


Figure 14: Ratio of ferritin subunit mRNA to β-actin mRNA. The mean ratio and standard error of the mean are given.

The mechanisms responsible for the regulation of ferritin mRNA turnover are not known and it is conceivable that increased translational activity may promote ferritin mRNA degradation. The turnover of many mRNAs can vary dramatically in response to a number of stimuli, and the half lives of various mRNAs range from 30 minutes to several days (Berger and Cooper, 1975; Raj and Pitha, 1981).

Other limitations of this study are the variations between subjects in terms of disease duration and severity, nutritional status and immunological response. In addition, it is conceivable that the adherence method of monocyte purification may have stimulated changes in ferritin gene expression.

3.3 INDUCED INFLAMMATION IN ANIMALS

3.3.1 Introduction

Previous studies investigating the mechanism of ferritin synthesis in turpentine-injected rats have used indirect methods, such as *in vitro* translation, to quantify ferritin mRNA content. The availability of cDNA probes for human and rat ferritin subunits now allows a more direct assessment of cellular ferritin mRNA content. This study was performed to directly investigate ferritin mRNA in animals during the early phase of increased ferritin synthesis caused by inflammation induced by turpentine injection. Splenic tissue was investigated as reticuloendothelial cells play a vital role in the hypoferraemia of inflammation (Noyes, 1960). In addition, hepatic tissue was studied in view of the hypothesis that ferritin is produced as an acute phase protein (Konijn and Hershko, 1977).

3.3.2 Methods

Approval for this study was obtained from the Animal Ethics Committee of the University of the Witwatersrand (protocol number 180/88). This was subject to the animals being maintained under general anaesthesia from the time of injection with turpentine until they were sacrificed, because of the pain of the resulting turpentine abscess. Male Sprague-Dawley rats with a weight of 250 g to 300 g were used. The rats were maintained on a standard rat feed and were not fasted prior to the experiment. Anaesthesia was induced in a halothane chamber, and the rats were injected with 0,5 ml turpentine into each hind leg. Anaesthesia was maintained in the halothane chamber using 100% oxygen and 0,5 to 1,2% halothane (Fluothane, ICI, South Africa). This inhalational anaesthetic has been shown not to

inhibit the acute phase response to inflammation (Simpson et al, 1987). The animals were repositioned intermittently during the period of anaesthesia. Four animals were exsanguinated by cardiac puncture at 3, 6 and 12 hours after injection. Two control groups were used without turpentine injection, one killed immediately after induction of anaesthesia and one after 6 hours of halothane anaesthesia.

Serum iron and total iron binding capacity were performed according to the method of the International Committee for Standardization in Haematology (Iron Panel) (1978 a&b). Splenic and liver tissue (50 to 100 mg) was obtained from each animal immediately after exsanguination and specimens were placed in 1 ml ice-cold guanidinium denaturing solution. These were homogenized in an Ultra-Turrex homogenizer and RNA was extracted as described above, and stored at -70°C. All RNA specimens were assessed by spectrophotometry and by agarose gel electrophoresis. RNA specimens (5 /ug) were slot-blotted and probed with the rat ferritin subunit probes as well as the β-actin probe. The amount of ferritin mRNA was compared by visual assessment of the northern blots and by quantitative scintillation counting of the ferritin and β-actin bands. Statisical analysis of this data was done by an analysis of variance, and student's t test.

3.3.3 Results

The results of sequential serum iron values revealed a significant hypoferraemic response in experimental rats compared to time 0 controls ($p < 0.01$) (figure 15). However, the hypoferraemia occured earlier than the 6 hours demonstrated by Konijn and Hershko (1977) and it can be seen that the halothane exposed controls rats also demonstrated significant hypoferraemia ($p < 0,05$). Halothane is known

to produce a pyrexial response in certain situations and it appears therefore that the halothane anaesthesia may have contributed in part to the inflammatory response. Being an inhalational anaesthetic, it is conceivable that halothane would have a more rapid effect than intramuscularly injected turpentine. The iron and ferritin changes investigated in this study may therefore be a result of both the turpentine-induced inflammation and an effect of halothane. Although slight changes in ferritin subunit mRNA were noted in halothane controls compared with time 0 controls, none reached statistical significance ($p > 0,10$). Halothane has been demonstrated not to increase hepatic total RNA synthesis in rats after 18 hours exposure at a concentration of 0,5% (Bastian, 1988).

In terms of the ferritin subunit mRNA content, no significant increase was noted in either hepatic or splenic tissue by visualization of the slot-blots (figures 16 & 17). Quantitative scintillation counting demonstrated no increase in ferritin subunit mRNA in inflammation and a significant decrease in hepatic L-ferritin mRNA content at 6 hours ($p < 0,05$) (figure 18). Splenic L-ferritin mRNA showed a downward trend, not reaching statistical significance (figure 19). An increase was noted in the ratio of the H-ferritin to L-ferritin mRNA on induction of inflammation (figure 20), suggesting differential regulation of the two subunits. This increase was not statistically significant ($p > 0,10$).

β-actin mRNA levels were measured with a view to using them as constitutively expressed controls (figure 21). Splenic levels did not vary throughout the experimental period, validating the use of this as a control in the investigation of monocyte RNA in section 3.2. However, hepatic levels of β-actin mRNA showed a marked increase on

induction of inflammation ($p = 0,001$), with no change noted in the halothane control. This is in accordance with previous work demonstrating increased hepatic actin synthesis in inflammation (Morrow et al, 1981). As the hepatic actin mRNA levels changed in this study, no constitutively expressed RNA was available as a control. However, constant amounts of total RNA were used in each slot-blot, so comparisons could be made between the different time-intervals.

3.3.4 Conclusions

No significant increase in ferritin subunit mRNA was demonstrated on induction of inflammation, suggesting a post-transcriptional mechanism for regulation of increased ferritin synthesis in this situation. This is consistent with the work of Schiaffonati and co-workers (1988) who found no increase in hepatic L-subunit mRNA in inflammation. The decrease in the L-ferritin mRNA subunit must be interpreted in the light of data indicating preferential synthesis of the L-ferritin subunit protein in inflammation (Schiaffonati et al, 1988). This suggests that a post-transcriptional shift of this mRNA to polyribosomes must occur. Although this may indicate a similarity with the iron-induced mechanism of ferritin synthesis (Aziz and Munro, 1986; Rogers and Munro, 1987), more recent data suggests that that iron causes a significant increase in the cellular levels of L-ferritin mRNA (White and Munro, 1988). Increased turnover of the ferritin mRNA, stimulated by inflammation, may account for the decreased L-subunit mRNA level noted in the present study. Changes in mRNA turnover have been demonstrated to play a role in regulation of

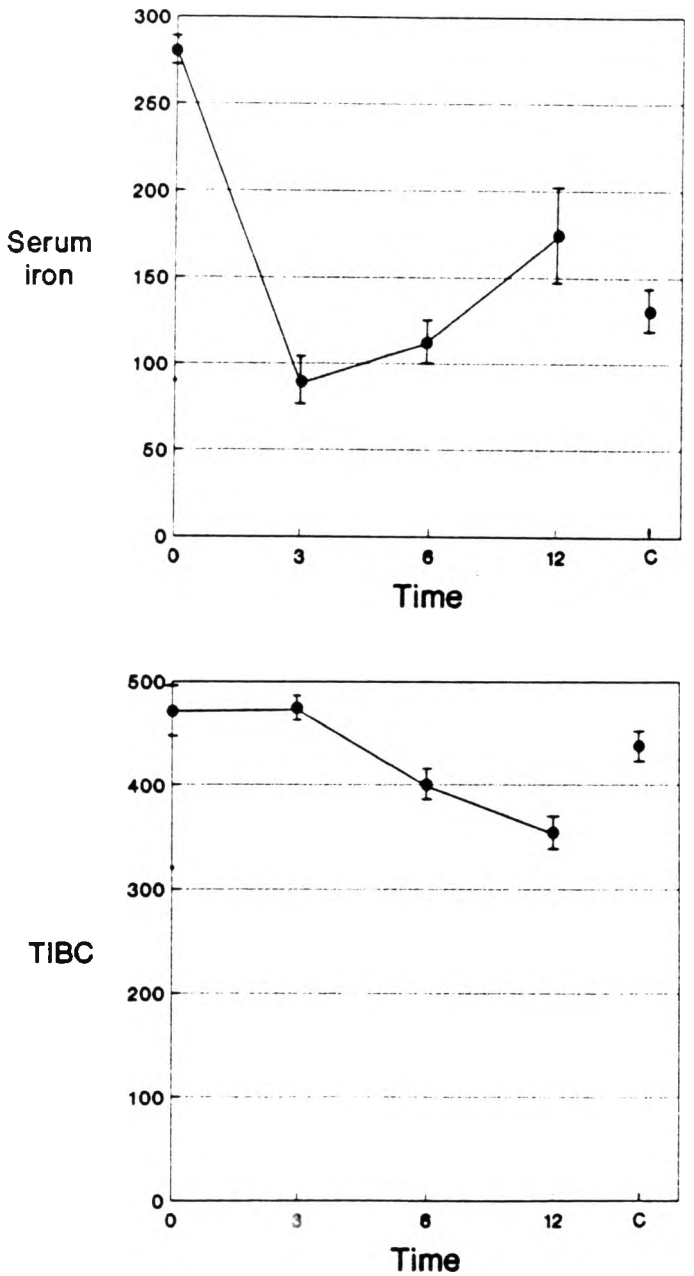


Figure 15: Graph of serum iron and total iron-binding capacity at various time intervals after turpentine injection and halothane exposed control (C) (mean and standard error of the mean).

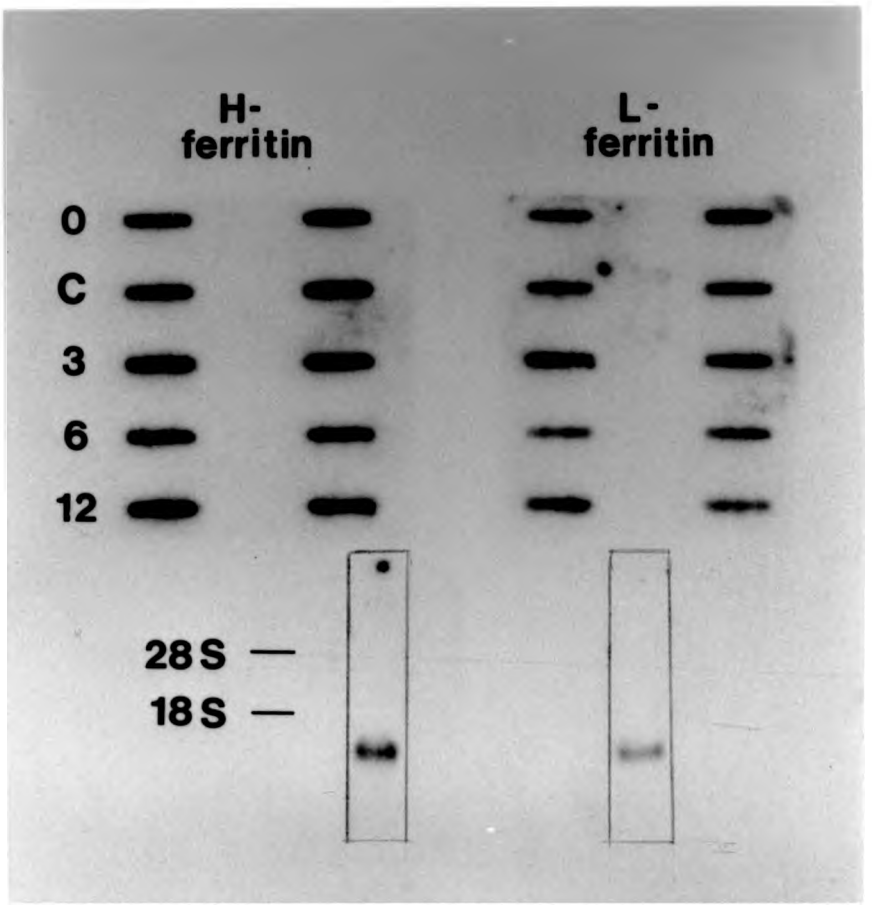


Figure 16: Autoradiograph of slot-blots of rat spleen RNA probed for H- and L-ferritin mRNA, with two specimens shown at each time interval (C = halothane exposed control). Corresponding northern blots, demonstrating integrity of the message, shown below.

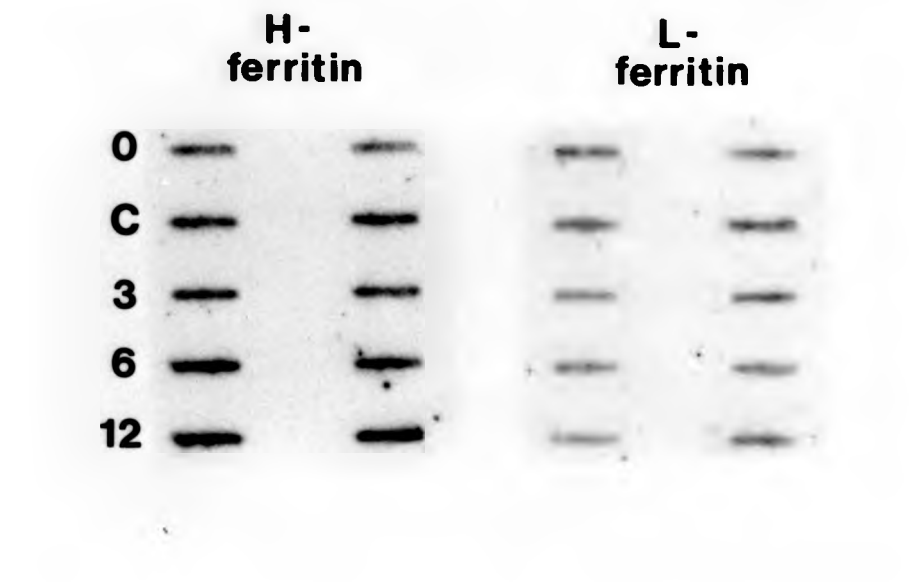


Figure 17: Autoradiograph of slot-blots of two specimens of hepatic RNA at each time interval, probed for H- and L-ferritin mRNA.

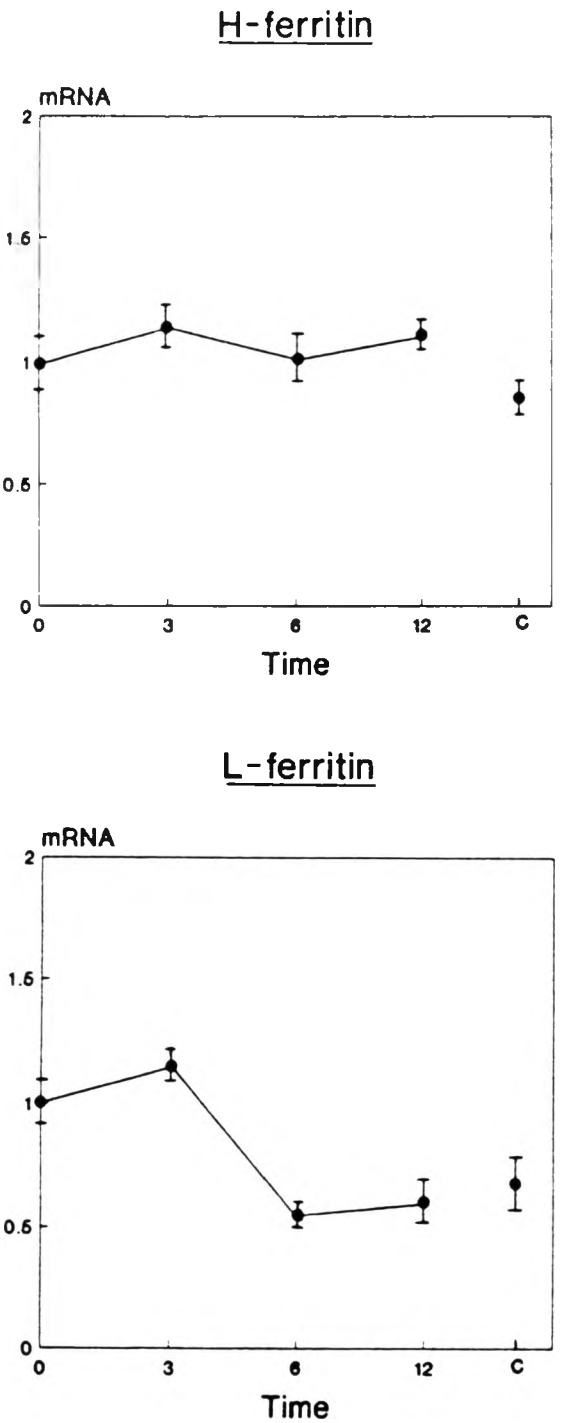


Figure 18: Mean and standard error of the mean of radioactive counts, representing the amount of H- and L- ferritin mRNA in hepatic tissue (values have been corrected such that the counts at time 0 = 1,0 ; 6 hour halothane-exposed control = C).

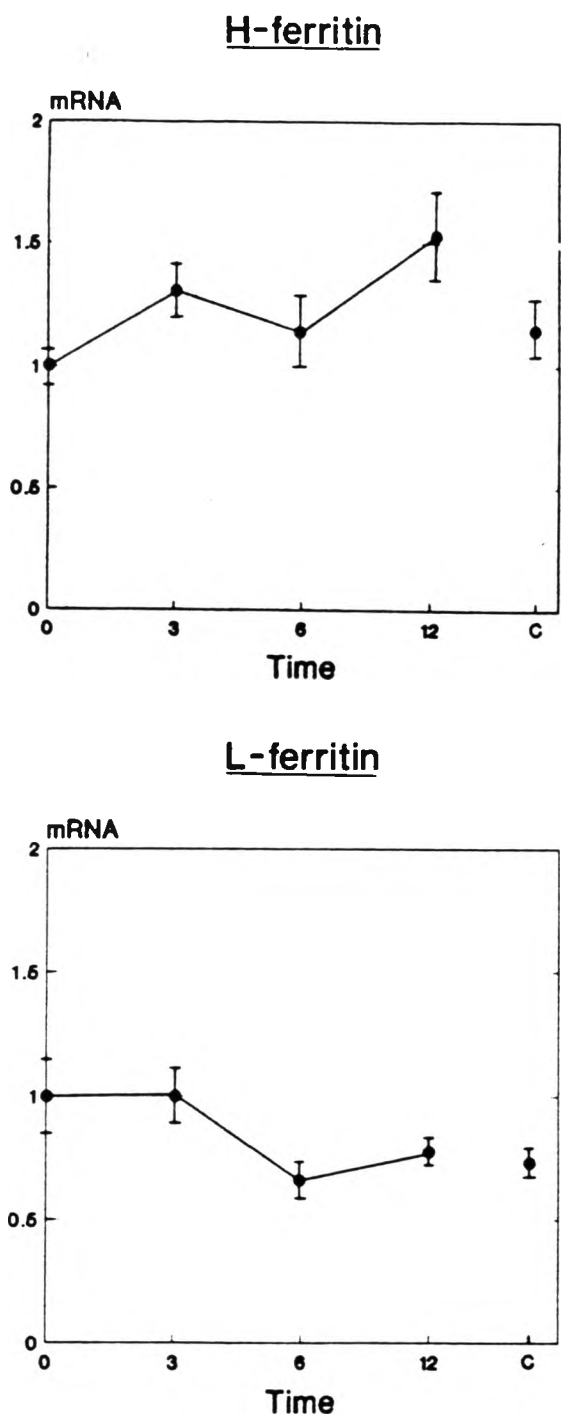


Figure 19: Mean and standard error of the mean of radioactive counts, representing the amount of H- and L- ferritin mRNA in splenic tissue (values have been corrected such that the counts at time 0 = 1,0).

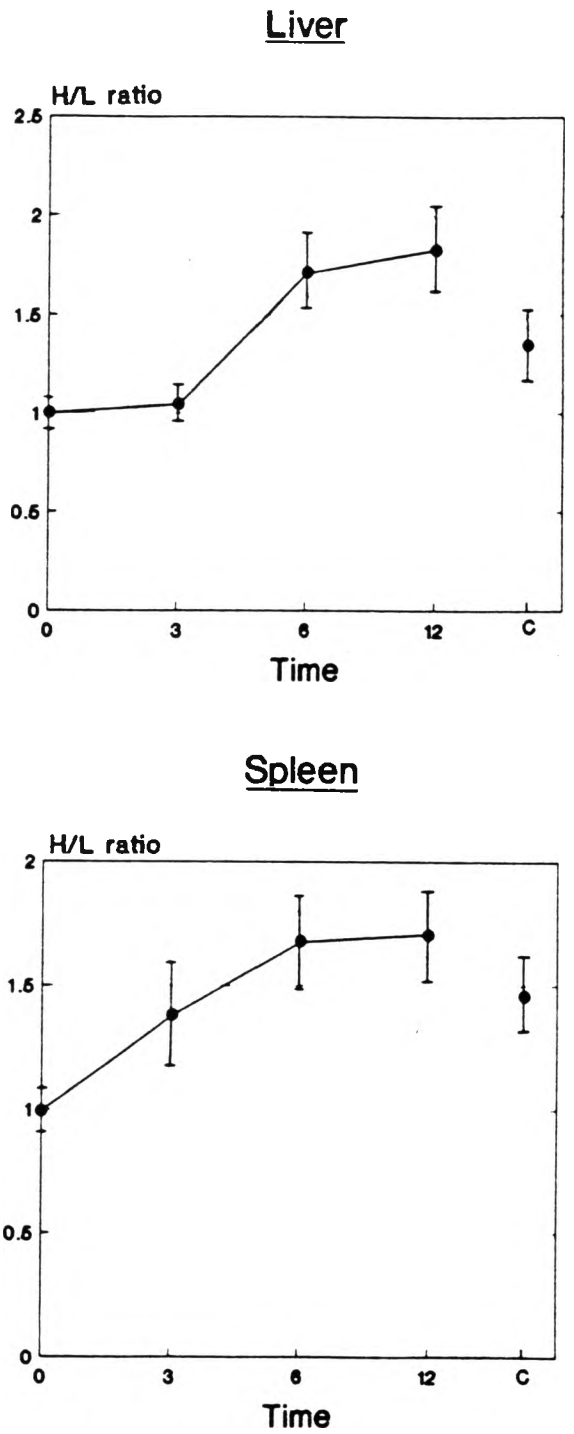


Figure 20: Ratio of H-ferritin to L-ferritin mRNA in hepatic and splenic tissue, corrected such that ratio at time 0 = 1,0 (mean and standard error of the mean).

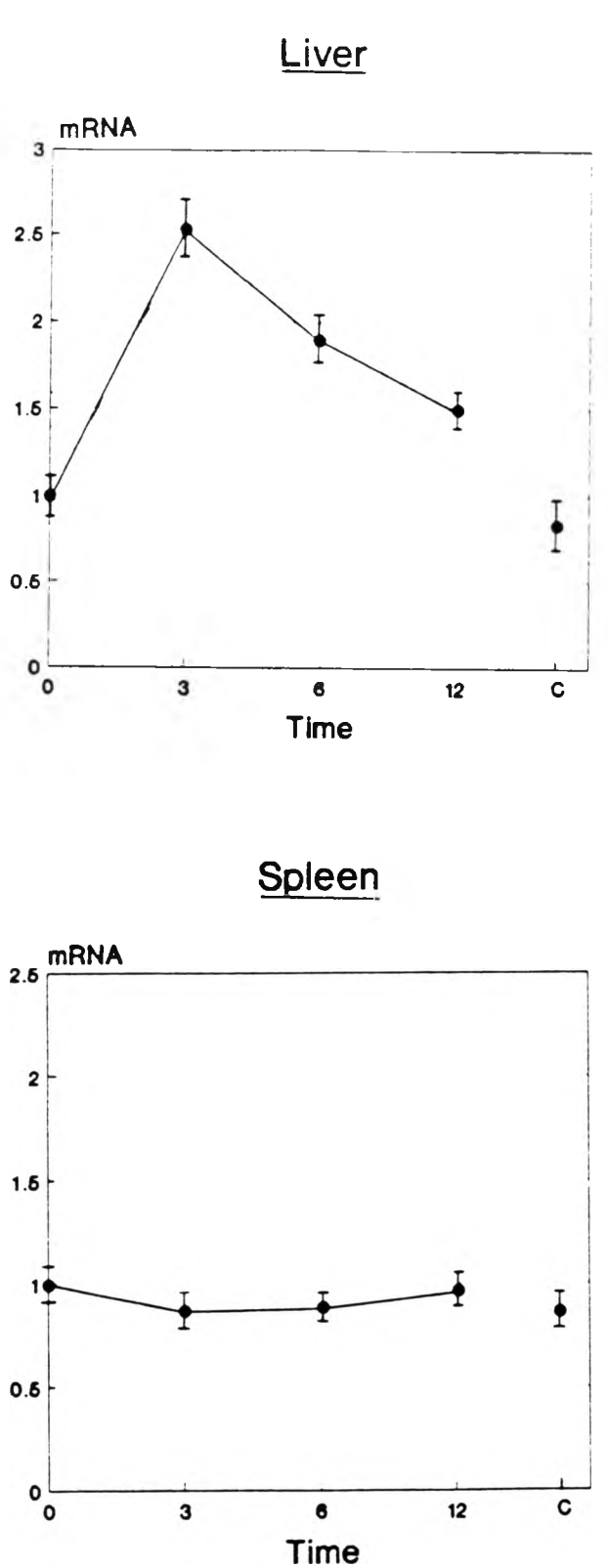


Figure 21: Mean and standard error of the mean of radioactive counts representing the amount of β-actin mRNA in hepatic and splenic tissue after induction of inflammation (values corrected such that time 0 = 1,0).

synthesis of a number of proteins. Hormonal factors may affect mRNA stability (Dani et al, 1985; Hamalainen et al, 1985) and a relationship may exist between protein synthesis and mRNA turnover for certain proteins (Brawerman, 1987). Structural features of the 3' and 5' untranslated regions of the mRNA molecule have been demonstrated to influence mRNA stability (Brawerman, 1987). Both ferritin subunit mRNAs have particulary long untranslated regions with highly conserved elements and this may play a role in gene expression (Murray et al, 1987). Changes in ferritin mRNA stability have been demonstrated in hepatoma cell lines (Costanzo et al, 1989) and on stimulation by iron (Mattia et al, 1989).

The trend towards an increase in the H- to L-ferritin mRNA ratio, demonstrated in both hepatic and splenic tissue, may result from the decrease in L-ferritin mRNA. Although no rise in H-ferritin mRNA was noted in the present study, a rise in the H/L ratio, probably due to increased H-subunit mRNA transcription, has been described in certain situations. This occurs where ferritin is synthesized for housekeeping use, such as differentiation of human leukaemia cell lines (Chou et al, 1986) and bullfrog reticulocytes (Dickey et al, 1987).

Although the mechanisms of ferritin mRNA expression in inflammation are not clear, the current data suggest that the two subunits are regulated by different mechanisms and that ferritin, unlike other acute phase reactants, is not synthesized by simple transcriptional means.

The increase levels of β-actin mRNA in inflammation are consistent with the data of Morrow and co-workers (1981), who demonstrated a marked increase in hepatic actin synthesis after administration of

lipopolysaccharide to mice. The absence of an increase in splenic β-actin mRNA validates this control for use with reticuloendothelial tissue, but for use with liver tissue a better control would be a specific ribosomal RNA probe.

3.4 INTRACYTOPLASMIC DISTRIBUTION OF mRNA

3.4.1 Introduction

Sections 3.2 and 3.3 investigated changes in the cellular content of ferritin mRNA on stimulation of reticuloendothelial cells by inflammation. The absence of a significant increase in ferritin mRNA levels is suggestive of a post-transcriptional mechanism of induction of ferritin synthesis, but changes in mRNA turnover may complicate this interpretation. It is conceivable that increased transcriptional activity may promote ferritin mRNA degradation. Direct demonstration of intracytoplasmic shifts of mRNA in response to inflammation would confirm the post-transcriptional mechanism of ferritin synthesis.

The question of changes in subcellular distribution of ferritin mRNA in inflammation was addressed by Konijn and co-workers (1981) who investigated hepatic ferritin synthesis in inflammation using the turpentine-injected rat. They found a biphasic rise in ferritin synthesis in hepatic cells, with an early peak at 6 hours after injection, dropping back to control levels by 12 hours. A later increase began at 24 hours and continued to rise at 48 hours. Haemopexin, another protein synthesized by the liver in response to inflammation, demonstrated only the late increase in synthesis. Using *in vitro* translation to assess ferritin mRNA content and subcellular distribution, they found that the early rise was associated with increased synthetic activity of their "cell sap" fraction. However, their methodology could not differentiate between increased amounts of ferritin subunit mRNA and increased affinity of this mRNA for polyribosomes. Their interpretation of these results was that the early phase was regulated at a post-trancriptional level. In addition, although all necessary precautions were taken, their

methodology does not allow for identification of partial degradation of mRNA.

The changes in intracytoplasmic distribution of ferritin mRNA induced by iron have been demonstrated in animal studies and in vitro by probing RNA with ferritin cDNA probes, after fractionating RNA on sucrose gradients (Aziz and Munro, 1986; Rogers and Munro, 1987). A pool of free mRNA was demonstrated toward the top of the gradient with ribosome-bound ferritin mRNA sedimenting further down the gradient. A shift to the ribosome-bound fraction occurred after stimulation of the cells with iron.

This study aimed to investigate changes in intracytoplasmic distribution of ferritin mRNA in reticuloendothelial cells after induction of inflammation. Splenic tissue from the turpentine-injected rat model was used, to demonstrate a time-course pattern of mRNA redistribution.

3.4.2 Methods

The anaesthesia and turpentine injection of Sprague-Dawley rats was performed as described in section 3.3.2 above. In addition, a number of control rats were studied when the problem of RNA degradation was encountered. After exsanguination by cardiac puncture, 50 to 100 mg of splenic tissue was placed in 2 ml of homogenizing buffer with RNAase inhibitors including vanadyl-ribonucleoside complexes (see section 2.9.2) and homogenized in an Ultra-Turrax homogenizer. A post-mitochondrial supernatant was layered on a 10 to 50% sucrose gradient and centrifuged for 4 hours at 26 000 rpm. The gradient was divided into 8 equal fractions and RNA was purified by phenol-chloroform extraction and isopropanol

precipitation. The fractions were electrophoresed in agarose gels, northern blotted onto nylon membrane and hybridized with ferritin subunit cDNA probes. Autoradiographs were exposed at -70°C for 6 to 36 hours.

3.4.3 Discussion

As discussed in section 2.9, RNA degradation is a significant problem when RNA is extracted without the use of guanidinium isothiocyanate, as required for this phase of the study. A marked reduction of degradation was obtained by the use of various RNAase inhibitors (figures 9, 10 and 11). Initial attempts at probing northern blots of fractionated splenic RNA resulted in no ferritin mRNA bands. Subsequent control experiments revealed the ferritin mRNA to be degraded, with hybridization of probe to a poorly defined band of smaller molecular weight than whole ferritin mRNA (figure 22). Significant degradation of similarly processed hepatic tissue did not occur. The splenic ferritin mRNA bands obtained were not of sufficient quality to interpret changes in mRNA distribution. In addition, very little mRNA could be demonstrated in the upper cytoplasmic fraction, possibly indicating that the ribosomal-bound mRNA was protected to some extent from degradation. A recent report published subsequent to the completion of this study demonstrated a post-transcriptional shift of L-ferritin mRNA to polyribosomes in the turpentine-injected rat (Campbell et al, 1989). However the published data did not include northern blots to indicate lack of mRNA degradation.

The problems of RNA isolation from mononuclear cells without guanidinium has been noted (Berger and Birkenmeier, 1979), compared

to hepatic tissue which appears to inhibit RNA degradation (Emtage et al, 1974). Although numerous control experiments with various RNAase inhibitors were performed (see section 2.9.1), undegraded RNA of sufficient quality could not be obtained after sucrose gradient centrifugation. As relatively small changes in mRNA distribution are expected, and the possibility occurs that some forms of ferritin mRNA (eg. ribosomal-bound) may be protected to some extent from enzymatic breakdown, absolute inhibition of degradation is necessary for valid results to be obtained. Further studies of the inhibition of RNAases would be required before a meaningful investigation of splenic mRNA redistribution could be performed.

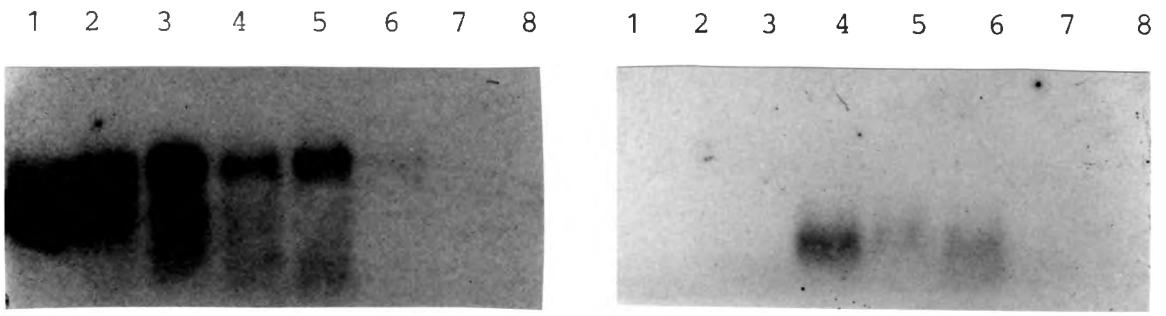


Figure 22: Autoradiograph of northern blots of hepatic and splenic RNA after fractionation on sucrose gradients, probed for L-ferritin mRNA (1 = lowest fraction, 8 = upper fraction)

3.5 CONCLUSIONS

Inflammatory states are associated with well documented changes in body iron metabolism, of which increased reticuloendothelial ferritin synthesis appears to play a central role. Ferritin synthesis is known to be stimulated by iron and this occurs by means of a post-transcriptional shift of mRNA from a free cytoplasmic pool to translationally active polyribosomes. The mechanism of the increased ferritin synthesis in inflammation has not been established.

The first part of the present study investigated ferritin mRNA content in circulating monocytes of patients with acute and chronic inflammatory disease, comparing them with normal controls. Although the characteristic changes in iron metabolism accompanying inflammation were demonstrated, no increase in the H- or L-subunit mRNA was noted. This indicates that in the steady state of established inflammation no transcriptional increase in ferritin mRNA occurs.

Animal studies were performed to investigate the acute changes in ferritin mRNA in rats with inflammation induced by turpentine injection. Follow up over a period of 12 hours revealed no increase in ferritin subunit mRNA in hepatic or splenic tissue, again indicating a post-transcriptional mechanism of ferritin synthesis. A decrease in the L-ferritin subunit mRNA was noted, resulting in a change in the H to L mRNA ratio. This suggests that changes in the stability of the ferritin mRNA may play a role in regulation of ferritin synthesis in inflammation. An interesting additional finding was that of an increase in the levels of β-actin mRNA in liver tissue of rats with induced inflammation.

Investigation of the mechanism of ferritin synthesis by assessing total ferritin mRNA levels may be complicated by changes in mRNA turnover. In order to directly demonstrate post-transcriptional shifts of mRNA, the intracytoplasmic distribution of ferritin mRNA was investigated, but no conclusive data was obtained.

The findings of these investigations indicate that in inflammatory states, reticuloendothelial ferritin synthesis occurs at a post-transcriptional level, with differential regulation of the two ferritin subunits.

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