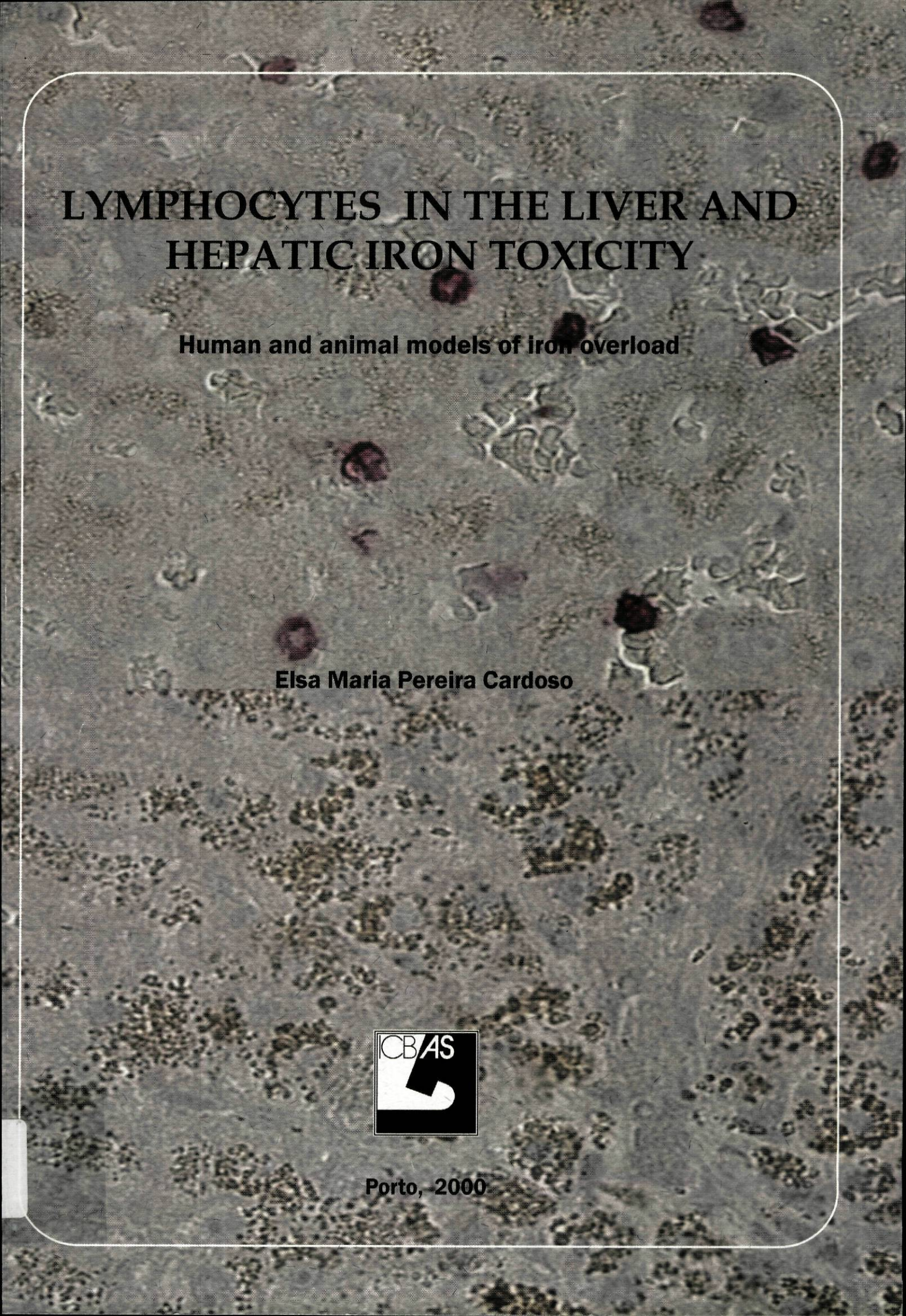


LYMPHOCYTES IN THE LIVER AND HEPATIC IRON TOXICITY

Human and animal models of iron overload

Elsa Maria Pereira Cardoso

Porto, 2000

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Cover: Liver sections from patients with hereditary haemochromatosis (HH) stained for the presence of $CD8^+$ cells. On the top, illustration of the presence of a high number (>100) of $CD8^+$ cells/mm^2; at the bottom illustration of low number (<20) of $CD8^+$ cells/mm^2; Paraffin sections stained by the APAAP method (Fast Red substrate); Original magnification ×250. [see chapter 3, page 40].

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LYMPHOCYTES IN THE LIVER AND HEPATIC IRON TOXICITY

Human and animal models of iron overload

ELSA MARIA PEREIRA CARDOSO

THESIS TO OBTAIN THE DEGREE OF DOCTOR IN BIOMEDICAL SCIENCES, SPECIALITY OF IMMUNOLOGY

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PORTO 2000

To my family
À minha família

To my friends
Aos meus amigos

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SUMMARY

Studies done in peripheral blood (PB) from patients with hereditary haemochromatosis (HH) have established that lymphocytes, in particular the $CD8^+$ cell subset may have a modifier role in iron homeostasis: HH patients with low numbers of $CD8^+$ PB lymphocytes have a more severe iron overload. Whether in HH patients there was an abnormal distribution between the blood and tissues of $CD8^+$ cells, or defective numbers occurred in affected tissues such as liver remained unknown. Because the gene of HH (*HFE*) was discovered shortly after this study had started, a further characterisation of the HH patients became possible, by genotyping them for the two main mutations found in the *HFE* gene (chapter 2). Then, the comparison between the numbers of $CD8^+$ cells in the blood and in the liver, and its association with iron status and liver damage, was evaluated in a group of HH patients selected with strict criteria, including homozygosity for the C282Y mutation in the *HFE* gene. This study showed that the number of $CD8^+$ lymphocytes in the PB and in the liver correlated positively (chapter 3). Hence, rather than an abnormal distribution of these lymphocytes, it appears that some HH patients have a general defect in numbers of $CD8^+$ cells. Neither age, cirrhosis or iron overload *per se* seem to be responsible for the low number of $CD8^+$ cells observed in some HH patients. Importantly, low numbers of $CD8^+$ cells in the liver were associated with higher iron stores and with liver damage. This substantiates that the "setting" of $CD8^+$ cell numbers may be an important indicator of the progression of iron overload in HH.

To investigate *in vivo* possible mechanisms involved in the interaction between lymphocytes and iron homeostasis, the presence of a marker of cytotoxicity (TIA-1) was evaluated within the $CD8^+$ cell subpopulation in the liver and an effort to analyse cytokine producing cells in the liver was undertaken. The results showed that the TIA-1 molecule is expressed in a broad range of liver $CD8^+$ cells, expression that did not correlate with iron stores or total number of $CD8^+$ cells. Thus, the possibility that a cytotoxic response to iron overload involving the TIA-1 molecule in HH has been excluded (chapter 3). The detection of local cytokine producing cells in liver tissue proved to be tecnhically very difficult, both by using immunohistochemistry and *in situ* hybridisation (appendix I). In part, this could be explained by the fact that HH patients have low numbers of lymphocytes in the liver (as it was shown for the $CD8^+$ cell subset, chapter 3).

Finally, in a study done with athymic and euthymic rats, fed with an iron-supplemented diet, a significant difference in iron distribution, measured as the liver to spleen ratio (of iron

accumulation) was found. This indicates that the thymus-derived cells may influence the pattern of tissue iron deposition (chapter 4).

RESUMO

Estudos feitos em sangue periférico de doentes com hemocromatose hereditária (HH) revelaram que os linfócitos, em particular a subpopulação $CD8^+$ pode desempenhar um papel modificador na homeostase do ferro: pacientes com HH e com poucos linfócitos $CD8^+$ no sangue periférico têm uma sobrecarga de ferro mais acentuada. Permanecia por examinar se nestes doentes existia uma distribuição anómala destas células entre o sangue e os tecidos, ou se o reduzido número de células $CD8^+$ ocorria também nos tecidos afectados, nomeadamente no fígado. Dado que o gene da HH (*HFE*) foi descoberto pouco tempo depois deste estudo ter sido iniciado, foi possível caracterizar melhor os doentes com HH, genotipando-os para as duas mutações principais do gene *HFE* (capítulo 2). Posteriormente, foi feita a comparação entre o número de células $CD8^+$ no sangue e no fígado, assim como a sua associação com os nívels de ferro e lesão hepática, num grupo de doentes com HH selecionados com base em critérios estritos, incluindo a homozigotia para a mutação C282Y no gene *HFE*. Este estudo mostrou que o número de linfócitos $CD8^+$ no sangue periférico e no fígado correlacionavam positivamente (capítulo 3). Assim, afigura-se provável que alguns doentes com HH têm um defeito geral nos números de células $CD8^+$, em detrimento de uma distribuição anormal destas células. Nem a idade, nem a cirrose ou a sobrecarga de ferro *per se* parecem ser responsáveis pelo reduzido número de células $CD8^+$ observado em alguns doentes com HH. De realçar que a existência de poucas células $CD8^+$ no fígado estava associada com uma sobrecarga de ferro maior e à lesão hepática. Estes resultados vêm reforçar a noção de que o número de células $CD8^+$ pode ser um indicador importante do desenvolvimento da sobrecarga de ferro em HH.

Com o intuito de investigar, *in vivo,* possíveis mecanismos envolvidos na interacção entre linfócitos e a homeostase do ferro, foi avaliada a presença de um marcador de citotoxicidade (TIA-1) na subpopulação de células $CD8^+$ do fígado, assim como foi feito um esforço para analisar células produtoras de citoquinas no fígado. Os resultados mostraram que a molécula TIA-1 é expressa num número de células $CD8^+$ largamente variável, e que não se correlacionava com a sobrecarga de ferro nem com o número total de células $CD8^+$. Assim, foi excluída a possibilidade de uma resposta citotóxica à sobrecarga de ferro em HH, envolvendo a molécula TIA-1 (capítulo 3). A detecção de células produtoras de citoquinas no fígado, tanto por imunohistoquímica como por hibridização *in situ*, revelou-se tecnicamente bastante difícil (Anexo I). Em parte, isto poder-

se-à explicar pelo facto dos doentes com HH terem poucos linfócitos no fígado (como foi evidenciado na subpopulação de células $CD8^+$, capítulo 3).

No estudo realizado com ratos atímicos e eutímicos, sujeitos a uma sobrecarga de ferro induzida por uma dieta rica em ferro, verificou-se uma diferença estatisticamente significativa na distribuição do ferro, medida como a razão baço – fígado (de acumulação de ferro). Este resultado indica que as células derivadas do tímo podem influenciar o padrão tecidular de deposição de ferro (capítulo 4).

RESUMÉ

Des études effectuées avec du sang périphérique de malades atteints d' hemocromatose héréditaire (HH) ont montré que les lymphocytes, en particulier la subpopulation $CD8^+$, pouvaient avoir un rôle déterminant dans l'homéostasie du fer: des malades atteints d' HH et possédant peu de lymphocytes $CD8^+$ dans le sang périphérique ont une surcharge en fer plus accentuée. Il restait à determiner si, chez les malades atteints d' HH, il existait une distribution anormale des cellules $CD8^+$ entre le sang et les tissues, ou si il existait un nombre réduit de cellules $CD8^+$ dans les tissues affectés, notamment le foie. Le gène de l'HH (*HFE*) ayant été découvert peu de temps après le début de cette étude, il a été possible de mieux caractériser les malades atteints d'HH, par génotypage des deux mutations principales du gène *HFE* (chapitre 2). La comparaison entre le nombre le cellules $CD8^+$ dans le sang et le foie, ainsi que son association avec les niveaux de fer et le degré de lésion hépatique, a ensuite été effectuée dans un groupe de malades atteints d'HH sélectionnés selon des critères stricts, y compris l'homozygotie pour la mutation C282Y dans le gène *HFE*. Cette étude a montré une correlation positive entre le nombre de lymphocytes $CD8^+$ dans le sang périphérique et dans le foie (chapitre 3). Ainsi, il paraît que quelques malades atteints d' HH présentent une anomalie générale dans le nombre de cellules $CD8^+$, plutôt qu'une distribution anormale de ces cellules. Ni l'âge, ni la cirrhose ni la surcharge en fer *per se* ne semblent être responsables du nombre réduit de cellules $CD8^+$ observé chez certains malades. La présence de peu de cellules $CD8^+$ dans le foie a été associée à une plus grande surcharge en fer et à la lésion hépatique. Ces résultats confortent l'hypothèse que le nombre de cellules $CD8^+$ peut être un indicateur important du developpement de la surcharge en fer dans l'HH.

Dans le but d'étudier *in vivo* des mécanismes possibles impliqués dans l'interaction entre les lymphocytes et l'homéostasie du fer, la présence d'un marqueur de cytotoxicité (TIA-1) dans la subpopulation de lymphocytes $CD8^+$ dans le foie a été évaluée, et un effort pour analyser des cellules productrices de cytokines dans le foie a été entrepris. Les résultats ont montré que la molécule TIA-1 est exprimée dans un nombre de cellules $CD8^+$ du foie très variable, et que son expression n'est pas corrélée ni avec la surcharge en fer ni avec le nombre total de cellules $CD8^+$. La possiblilité d'une réponse cytotoxique à la surcharge en fer dans l'HH impliquant la molécule TIA-1 a donc été exclue (chapitre 3). La détection de cellules productrices de cytokines dans le foie, soit par immunohistochimie soit par hybridation *in situ*, s'est révélée techniquement assez difficile (Annexe I). Ceci pourrait être expliqué en partie par le fait que les malades atteints d'HH

possèdent un nombre réduit de lymphocytes dans le foie (comme cela a été mis en évidence pour la subpopulation de cellules $CD8^+$, chapitre 3).

Finalement, au cours d'une étude réalisée avec des rats athymiques ou euthymiques, ayant subi une surcharge martiale induite par un régime riche en fer, une différence statistiquement significative dans la distribution du fer a été observée, mesurée comme le rapport rate/foie (d'accumulation du fer). Ce résultat montre que les cellules dérivées du thymus peuvent influencer l'accumulation du fer dans les tissus (chapitre 4).

ABBREVIATIONS

ABC-HRP	avidin-biotin-complex-horse-peroxidase
ACD	anaemia of chronic disease
AEC	3-amino-9-ethylcarbazole
ALD	alcoholic liver disease
ALT	alanine transaminase
APAAP	alkaline phosphatase-anti-alkaline phosphatase
APC	antigen-presenting cell
β_2m	β_2-microglobulin
BT	biotinyl tyramide
CD	cluster of differentiation
CTL	cytotoxic T lymphocytes
DAB	diaminobenzidine
DMT1	divalent-metal transporter 1
DTT	dithiotreitol
EBSS	earls buffered salt solution
ECM	extracellular matrix
Fab	antigen-binding fragment
FasL	Fas ligand
Fc	crystallisable fragment
GMP-17	granule membrane protein-17 kDa
H_2O_2	hydrogen peroxide
HCV	hepatitis C virus
HFE	haemochromatosis gene
HH	hereditary haemochromatosis
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNE	4-hydroxynonenal
HPLC	high-performance liquid chromatography
HRP	horse peroxidase
ICAM-1	intracellular adhesion molecule-1
IEL	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRP	iron responsive protein
IRE	iron responsive element
LP	lamina propria
LPS	lipopolysaccharide
mAb	monoclonal antibody
MDA	malondialdehyde
MHC	major histocompatibility complex
MIC	MHC class I chain related
NK	natural killer
NMS	normal mouse serum
NO	nitric oxide

Nramp	natural resistance-associated macrophage protein
NRS	normal rabbit serum
NTBI	non-tranferrin bound iron
$O^{\bullet}_2^-$	superoxide
$^{\bullet}OH$	hydroxyl radical
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
RAG	recombination-activating gene
RE	reticuloendothelial
SA-HRP	streptavidin horse peroxidase
SEB	staphyloccus enterotoxin B
SD	standard deviation
SI	small intestine
SOD	superoxide dismutase
TBS	tris-buffered saline
TCR	T cell receptor
Tf	transferrin
TIBC	total iron binding capacity
TNF	tumour necrosis factor
TfR	transferrin receptor
TSA	tyramide signal amplification

CHAPTER 1 - GENERAL INTRODUCTION

IRON HOMEOSTASIS AND TOXICITY

Systemic iron homeostasis

Total body iron content is regulated by controlling the level of iron absorption from the diet in the small intestine and factors that are known to affect the uptake of this metal are *storage iron status*, *erythropoiesis activity* and *hypoxia* (Finch and Huebers, 1986). In addition, no regulatory mechanism seems to exist for the excretion of iron. This is limited to losses occurring as a consequence of the shedding of epithelial cells from the intestinal and urinary tracts and from the skin and small amounts of intestinal bleeding. Essential to systemic iron homeostasis is the recycling process whereby iron from senescent and damaged erythrocytes, which are removed from circulation by the reticuloendothelial (RE) system (macrophages in the spleen, liver and bone marrow), is reutilized for synthesis of new haemoglobin (Finch et al., 1970) (Figure 1.1). Hence, iron homeostasis is characterised by a limited external exchange and by an efficient reutilization of iron from internal sources. Dietary iron is absorbed by the small intestine and is incorporated into serum transferrin (Tf). This protein that binds iron in the ferric form, has a central role in this recycling process. Transferrin carries iron to meet tissue needs throughout the body and is the main source of iron for erythroid bone marrow and other dividing cells. Although only 3 to 5 mg of iron are associated with transferrin in the serum of a normal adult individual, the

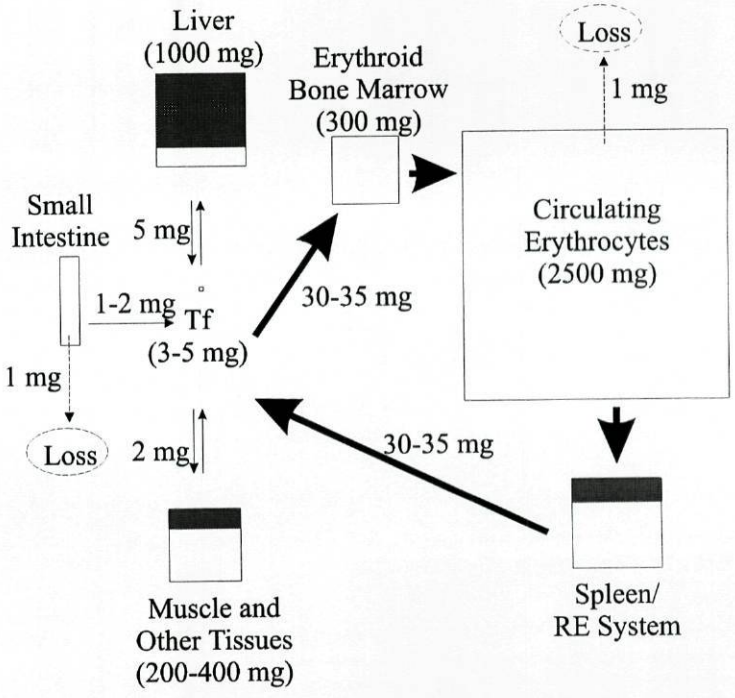


Figure 1.1 Schematic representation of human systemic iron homeostasis. Numbers on the arrows represent the approximate amounts of iron, in mg per day, involved in the traffic of iron. Numbers in parenthesis are the approximate total iron stores of the indicated tissues. A significant amount of iron is stored in ferritin (grey areas). Abbreviations: Tf, transferrin; RE, reticuloendothelial [The estimates of the size of iron compartments and the magnitude of iron movement shown in the figure are derived from Kuhn, 1994 and Brittenham, 1994].

flux through this pool is 30 to 35 mg per day, of which approximately 1-2 mg is derived by absorption and the remainder by reutilization. Thus, compared to the total iron pool present in various tissues, a small amount of iron is absorbed. This is normally sufficient to compensate for equally small losses of iron. The major proportion of the iron is present in the haemoglobin of circulating erythrocytes (approximately 2500 mg of iron) where it transports oxygen. The remainder of functional iron is found as myoglobin iron in muscle and as iron-containing or iron-dependent enzymes throughout the cells of the body (200-400 mg of iron). Most storage iron is held in hepatocytes and macrophages in the liver, bone marrow, spleen and muscle (Brittenham, 1994).

Iron toxicity

Molecular mechanisms of iron toxicity

An important feature of the chemistry of iron is its variable oxidation states (+2 and +3), which allows iron to participate in single-electron transfer reactions. The hydrated ferrous ion Fe^{2+} can reduce hydrogen peroxide, H_2O_2, in a one-electron reaction to generate *hydroxyl free radical*, $^{\bullet}OH$ (1); *superoxide* ($O^{\bullet}_2^-$) can reduce certain ferric chelates (2). The sum of reactions (1) and (2) is known as the iron-catalysed Haber-Weiss reaction (3).

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}\mathbf{OH} + OH^-$ (FENTON REACTION)

(2) $Fe^{3+} + O^{\bullet}_2^- \rightarrow Fe^{2+} + O_2$

(3) $O^{\bullet}_2^- + H_2O_2 \xrightarrow{Fe\ Catalyst} O_2 + {}^{\bullet}\mathbf{OH}$ (HABER-WEISS REACTION)

The hydroxyl free radical $^{\bullet}OH$, like other free radicals, is an extremely reactive species and highly non-selective. It can react very rapidly with almost every type of molecule, namely nucleic acids, lipids and proteins. Reacting with DNA they lead to *single-strand nicks* or *oxidative damage*, such as the formation of 8-hydroxyldeoxyguanosine (Imlay et al., 1988; Pryor, 1988). The induction of strand breakage in DNA has been associated with promotion events in chemically induced carcinogenesis (Hartley et al., 1985), and oxidative mutagenesis seems to be caused by misreading of modified bases and adjacent residues in DNA templates containing 8-hydroxydeoxyguanosine (Kuchino et al., 1987; Wood et al., 1990). Hydroxyl free radicals may also attack unsaturated lipid molecules (LH), leading to lipid peroxidation (Figure 1.2). Hydrogen abstraction leaves behind a carbon-centred free radical lipid molecule ($L^{\bullet}$), which reacts rapidly

with O_2 to form a lipid peroxyl free radical ($LO^{\bullet}_2$). The latter attacks another unsaturated lipid forming a lipid hydroperoxide (LOOH) and a lipid free radical ($L^{\bullet}$) to propagate the reaction. A complex mixture of lipid peroxides is formed by free radicals in this way. In the presence of reactive iron-complexes, lipid peroxides can be decomposed to form alkoxyl ($LO^{\bullet}$) and peroxyl ($LO^{\bullet}_2$) free radicals. Many breakdown products resulting from lipid peroxidation include reactive aldehydes, MDA and HNE. These products can then react with proteins forming adducts, which may inhibit the function of proteins.

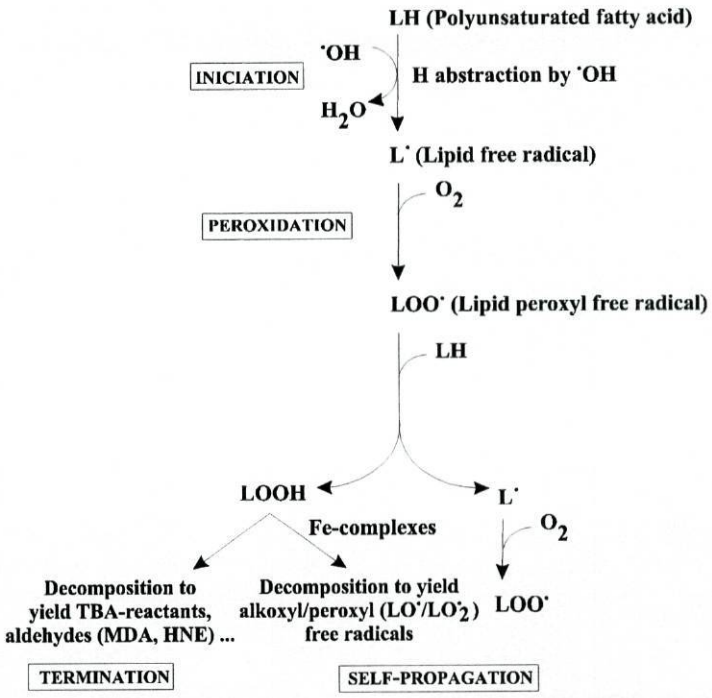


Figure 1.2 Pathway of lipid peroxidation of polyunsaturated fatty acids initiated by the hydroxyl radical [Adapted from Britton, 1996].

Iron-induced oxygen free radicals, by promoting lipid peroxidation or attacking DNA, lead to damage of several cellular constituents namely, lysosomes, mitochondria, plasma membrane, endoplasmatic reticulum and nucleus (reviewed by Britton 1996), resulting in wide-ranging impairment in cellular function and integrity. In the liver, for instances, the iron-induced oxidative stress can lead to hepatocellular necrosis, known as sideronecrosis. The necrotic hepatocytes are phagocyted by adjacent Kupffer cells, which may become activated (Stal et al., 1995) and start secreting pro-inflammatory cytokines. Cytokines can activate stellate cells, which normally store vitamin A but that following activation are transformed in myofibroblasts and produce collagen type 1, a major product of fibrosis (Friedman, 1990). That hepatic stellate cells are likely to be activated by paracrine signals arising from neighbouring cells was also shown in a rodent model of iron-associated fibrogenesis (Montosi et al., 1998). However, iron-induced fibrosis can occur in the absence of hepatocellular necrosis (Houglum et al., 1994). This suggests that sub-lethal lipid peroxidation might provide a direct non-necroinflammatory stimulus to fibrogenesis. In fact, reports of stimulation of collagen gene transcription by acetaldehyde have supported this hypothesis. In 1989, Chojkier and colleagues showed that in cultured human fibroblasts, lipid peroxidation was associated with increased production of collagen and procollagen mRNA

(Chojkier et al., 1989). These results have been reproduced later on human hepatic stellate cells (Parola et al., 1993). Potential mediators of this effect include lipid-derived free radicals and reactive aldehydes generated during lipid peroxidation in hepatocytes, all of which are freely diffusible across membranes (Esterbauer et al., 1987).

Defence systems, which prevent or correct iron-induced oxidative stress

As described above, iron can be an important promoter of free-radical reactions, which convert less-reactive to more-reactive species. Hence, living organisms have developed defence systems that prevent or correct iron induced oxidative stress: a strict iron *sequestration* and *compartmentalisation* of iron compounds, which are capable of catalysing reactions with molecular oxygen; different *enzymes* and *substances* which can remove reactive oxygen species. Thus, to limit the participation of iron in reactions leading to aggressive oxidation formation, the body takes great care to sequester iron in poorly or non-reactive transport and storage forms, such as transferrin, lactoferritin, ferritin and haemosiderin. Moreover, haemoglobin is transported inside erythrocytes, and the metabolic oxidation (whereby O_2 is reduced to H_2O, in a process that includes intermediary species such as $O^{\bullet}_2^{-}$, H_2O_2 and $^{\bullet}OH$) occurs inside an organelle (the mitochondria), both compartments that are rich in the antioxidant enzymes, catalase and superoxide dismutase (SOD). Several other substances can also function as antioxidants, namely vitamin C, vitamin E, uric acid, billirubin and sulfhydryl groups that can inactivate oxyradicals (Halliwell, 1990).

Hereditary haemochromatosis

Hereditary haemochromatosis (HH) is a genetic disease whereby iron homeostasis is disregulated. It is the most common autosomal recessive disorder, affecting approximately 1 in 200 to 1 in 400 Caucasian individuals (reviewed by Merryweather-Clarke et al., 1997). The gene mutated in HH (*HFE*) encodes a protein homologous to non-classical MHC class I molecules (Feder et al., 1996). In some parts of the world, between 69% to 100% of HH patients are homozygous for a mutation (C282Y) that converts a cysteine to a tyrosine (Cuthbert, 1997). This mutation eliminates a disulphide bond in the α3 domain of HFE, preventing association with β2m and cell-surface expression in cell-cultured models (Feder et al., 1997; Waheed et al., 1997). A second mutation that converts a histidine to an aspartate, increases the relative risk of developing haemochromatosis in subjects that are heterozygous for the C282Y mutation (Feder et al., 1996; Beutler et al., 1996). The HFE molecule has been shown to interact with the transferrin receptor (TfR) and to interfere with iron uptake into the cells (reviewed by Aisen et al., 1999). Although,

the HFE-TfR complex suggests a binding site for transferrin on TfR (Bennett et al., 2000), the stoichiometry of TfR-HFE on the cell surface, and how it influences Fe-Tf binding to TfR, remain to be further investigated.

Nevertheless, HH is characterised by a failure in regulating iron absorption, which leads to iron accumulation first in the transferrin pool, and later in several organs resulting in diverse pathologies of the liver (cirrhosis and hepatocarcinoma), pancreas (diabetes), joints (arthropathia), heart (arrithmias and cardiomyopathy) and hypophysis (hypogonadism) (Powell et al., 1994). The iron-binding capacity of transferrin in the plasma of iron-overload patients is often exceeded, so that non-tranferrin bound iron (NTBI) is present, mainly bound to citrate (Grootveld et al., 1989). This NTBI can accelerate lipid peroxidation (Gutteridge et al., 1985). Fibrogenic process is initiated at critical threshold levels of hepatic iron overload (Bassett et al., 1986), and if left untreated, the iron accumulation eventually leads to cirrhosis. HH patients with long-standing cirrhosis have an increased risk of developing hepatocellular carcinoma (Niederau et al., 1985). Despite the discovery of the HH gene, the clinical expression of the disease varies greatly and other factors are likely to interfere with the susceptibility to this disease among caucasians.

EMERGING FUNCTIONS FOR THE IMMUNE SYSTEM

The immune system is a complex and dynamic cellular network. It defends the organisms, both against challenges coming from the outside world and the inside environment. The prime task of the immune system has been considered to mediate protection against microbes and viral pathogens, coming from the external world (Ploegh, 1999). The concept that the immune system may also have a surveillance role against challenges coming from within the organism, such as tumour cells, was first formulated by Thomas in 1959.

Current discoveries are expanding the type of "antigens" that the immune system can "see", as well as the type of responses it may carry out. Hence, recent studies showed that lymphocytes not only recognise peptides in the context of MHC class I and II molecules (Germain and Margulies, 1993), but are also able to recognise lipids and lipoglycan compounds presented by non-classical MHC class I molecules such as CD1 (Melian et al., 1996). Stenger and colleagues recently demonstrated that double negative ($CD4^-CD8^-$) CD1-restricted T cells participate in the elimination of infected macrophages via interaction between Fas and Fas ligand, whereas $CD8^+$ T cells used a granule-dependent mechanism (Stenger et al., 1997). Thus, different subsets of lymphocytes, albeit recognising the same molecule (CD1), exhibit their cytotoxic function through different mechanisms. In addition, numerous other functions of CD1d-restricted lymphocytes are only emerging (Mendiratta et al., 1997; Prussin and Foster, 1997; Cui et al., 1997; Wilson et al.,

1998). Moreover, other lymphocytes, namely TCR$\gamma\delta^+$ T cells were shown to recognise non-classical MHC class I molecules, MICA and MICB (independent of peptide cargo), which are the stress-induced molecules expressed on epithelial cells and tumours (Groh et al., 1996; 1998; 1999). This interaction leads to activation of these lymphocytes, which appears to be crucial in maintaining the integrity of the gut epithelium by eliminating infected or damaged epithelial cells, and in controlling tumour growth. The observation that non-classical antigen presenting cells (APCs) may stimulate lymphocytes and regulate immune responses has been demonstrated not only with intestinal epithelial cells (Mayer, 1998), but also in other epithelial cells such as keratinocytes (Nickoloff and Turka, 1994) and hepatocytes (Paroli et al., 1994; Bertolino et al., 1998). Indeed, hepatocytes do not seem to be merely "passive" target cells, but important "immunoregulator" cells which can influence their own environment, by their ability to express receptors for immune mediators, as well as immune adhesion molecules and activation markers (Volpes et al., 1992).

Thus, different subpopulations of lymphocytes after encountering an "antigen" may become activated, start proliferating, become cytotoxic or secrete different types of cytokines, depending on the environment where this process occurs (Mosmann and Sad, 1996). Cytokines are mediator molecules, which effect many metabolic functions in different cells, not only inside but also outside the immune system, maintaining or restoring their physiological homeostasis. In the liver, cytokines have been shown to influence, among others, amino acid, protein, lipid, carbohydrate and mineral metabolisms (Andus et al., 1991). The two key words describing cytokine biological action are *pleiotropy* and *redundancy*. Thus, cytokines are able to exert different biological effects on different cell types and at the same time they can act as a positive and a negative signal, depending on the cell type and the metabolic state of the target cells. The response of the target cells to a given cytokine is determined by the expression of the cytokine receptor and/or the nature of the link between the receptor and the signal transduction pathways of the target cells. Conversely, several cytokines may have overlapping functions, i.e. cytokines, through different receptors, may evoke identical biological responses. In this case, multiple receptors may couple to the same signal transduction pathway via common transducers or effectors. Knockout mice are revealing that only few cytokines are absolutely essential for life or even for individual cellular functions (Taverne, 1993). These observations, rather than suggesting that individual cytokines are unimportant, suggest that important cellular functions are usually backed up in a "fail-safe" mechanism where one cytokine can compensate for the loss of another. Given their pleiotropy, cytokines usually act within close range in an autocrine or paracrine fashion in order to ensure specificity in the response. Close cell-to-cell contact and directed cytokine release, not only secures sufficient secreted amounts, but also minimises detrimental stimulation of by-stander cells

(Kupfer et al., 1991). Tolerant lymphocytes, which are commonly seen as inert cells, in spite of not proliferating, have been shown to survive for long periods *in vivo* and to secret interleukin-10 (IL-10). Thus, tolerance does not seem to be an absence of functional activity and rather appears to be associated with modifications of behaviour conferring important regulatory functions on tolerant T cells (Tanchot et al., 1998). Moreover, besides the cellular immune responses of $CD8^+$ lymphocytes being well recognised as cytotoxic ones (Berke, 1995), a suppressive response for intestinal $CD8^+$ lymphocytes has also been suggested (Mayer, 1997). Another example of non-cytotoxic $CD8^+$ T cell function has been observed in human immunodeficiency virus (HIV) infection and it seems to be mediated by an anti-viral factor (Levy et al., 1996).

Looking at the immune system from this perspective it appears that new potential functions for this system may emerge in the near future. It was within such a broaden conception of the immune system that in 1978, de Sousa postulated a role for the circulation of lymphocytes in the surveillance of iron-induced toxicity (de Sousa, 1978a). This metal, in spite of being one of the essential chemical elements of life, can also be very toxic to the cells and consequently to the body, as mentioned in the first part of this chapter. By promoting the formation of highly reactive oxygen free radicals, iron induces irreversible damage, especially to DNA and to cellular membrane lipids, a phenomenon termed "oxidative stress". Therefore, a tight surveillance system for iron homeostasis is essential.

A ROLE FOR THE IMMUNE SYSTEM IN REGULATING IRON HOMEOSTASIS AND IRON-INDUCED TOXICITY

The hypothesis of considering iron as a target of immune surveillance (de Sousa, 1978a) came along with evidence from earlier studies that lymphocytes migrate to and accumulate in tissues with high iron content, as well as the knowledge that lymphocytes participate in the synthesis and recognition of iron-binding proteins (de Sousa et al., 1978b). During the last two decades, it has become clear that the immune system has a role in the iron metabolism. The development of anaemia (anaemia of chronic disease, ACD), associated with low plasma iron, tranferrin, transferrin saturation and increased iron deposition in macrophages, has been strongly linked to immune effector functions (reviewed by Weiss et al., 1995; Weiss, 1999). In hereditary haemochromatosis (HH), a link between the immune system and iron metabolism has also been observed (reviewed by de Sousa et al., 2000).

The cells of immune system

Several studies in HH patients indicate that lymphocytes, in particular $CD8^+$ T cell subsets, play a role in the regulation of iron accumulation. Abnormalities in the relative proportions of the two major lymphocyte populations, $CD4^+$ and $CD8^+$ cells, were found in some HH patients (Reimao et al., 1991, Porto et al., 1997). The relative proportion of these two subpopulations (CD4/CD8 ratio) correlated significantly with the clinical expression of the disease and the severity of iron overload, in the context of HLA-A3 (Porto et al., 1997). Moreover, the abnormal ratios were due to abnormally low percentages of $CD8^+$ T cells (Porto et al., 1997), and both the CD4/CD8 ratio as well as their absolute numbers were not corrected when normal iron status was achieved, following intensive treatment. This finding ruled out the possibility that the low numbers of $CD8^+$ T cells were secondary to iron overload (Porto et al., 1994; 1997). This is also supported by *in vitro* studies showing that iron overload does not effect $CD8^+$ T cells (Santos and de Sousa, 1994; Arosa and de Sousa, 1995). In addition, HH patients with high CD4/CD8 ratio demonstrated a faster re-entry of iron into the transferrin pool after intensive phlebotomy treatment (Reimao et al., 1991). Recently, it has also been shown that HH patients with the highest iron retention values (more than 75%) demonstrate the lowest percentages of peripheral $CD8^+$ T cells (Moura, 1997a). Characterisation of the T cell repertoire in peripheral blood revealed abnormally low percentages of $CD8^+V\beta6.7^+$ cell subset in HH patients, particularly in those with cirrhosis (Cabeda et al., 1995). HH patients showed also a decreased CD8-$p56^{lck}$ activity (Arosa et al., 1994). Moreover, this defective CD8-associated $p56^{lck}$ activity could not be attributed to iron overload, since $p56^{lck}$ activity is regulated by iron in the CD4 but not in the CD8 cell subset (Arosa and de Sousa, 1995). The kinase $p56^{lck}$ is involved in signal transduction in T lymphocytes (Molina et al., 1992). The observed decreased CD8-$p56^{lck}$ activity in HH patients may thus be related to an "activated" $CD8^+$ T cell phenotype, since HH patients presented a higher percentage of $HLA\text{-}DR^+$, but not $CD45RO^+$ cells, among $CD8^+$ T lymphocytes, relative to controls (Arosa et al., 1997). Furthermore, within the $CD8^+$ T cell subpopulation, an increased percentage of $CD8^+CD28^-$ T lymphocytes, with a corresponding reduction in the percentage of $CD8^+CD28^+$ T lymphocytes, was present in HH patients, namely in those carrying the HLA-A3 allele (Arosa et al., 1997). CD28 expression was normal in $CD4^+$ T lymphocytes, however. Moreover, functional studies showed that $CD8^+$ cytotoxic T lymphocytes (CTL) from HH patients exhibited a diminished (approximately two-fold) cytotoxic activity, when compared with $CD8^+$ CTL from healthy controls (Arosa et al., 1997). Altogether, the accumulated evidence demonstrates that abnormalities in lymphocytes, in special in $CD8^+$ T cells, are present in HH patients, and more important, that some of those abnormalities are associated with a more severe clinical expression of the disease.

We have already mentioned that macrophages of the reticuloendothelial (RE) system play a central role in iron metabolism through the recycling of senescent erythrocytes. In HH iron is preferentially accumulated in hepatocytes, while iron accumulation in macrophages occurs only in later stages of the disease (Powell et al., 1996). Contributing to this low intracellular iron content observed in RE cells and monocytes in HH patients, may be an enhanced release of iron or ferritin observed in these cells *in vitro* (Fillet et al., 1989; Moura, 1997b; Flanagan et al., 1989). HH monocytes demonstrated an increased activity of the iron responsive protein (IRP), which regulates cellular iron metabolism through co-ordinate control the expression of ferritin and transferrin receptor, compared with normal controls (Cairo et al., 1997). IRP activity could be decreased *in vitro* following manipulation of iron levels, indicating that the increased activity of the IRP in HH monocytes was not due to an inherent defect of its control, but rather a consequence of low intracellular iron levels in those cells. Furthermore, HH monocyte preparations showed an impaired release of tumour necrosis factor-α (TNF-α) upon stimulation (Gordeuk et al., 1992).

Animal models have been used to evaluate the effect of absence of lymphocytes on iron-induced toxicity. Homozygous $\beta_2$microglobulin knockout mice (β_2m -/-), who show a faulty expression of MHC class I molecules at the surface, and consequently are deficient in $CD8^+$ $TCR\alpha\beta^+$ T cells (Zijlstra et al., 1990), were first reported to develop iron overload indistinguishable from that observed in human HH by de Sousa et al. (1994). This observation was confirmed subsequently by others, who predicted a role for class I genes of the major histocompatibility complex in iron metabolism (Rothenberg and Voland, 1996). Later the haemochromatosis gene (*HFE*), which turned out to be a non-classical MHC class I molecule, was identified (Feder et al., 1996). *HFE* -/- mice develop iron overload typical of HH, confirming the role of this molecule in iron metabolism (Zhou et al., 1998). Reconstitution of the iron overload β_2m -/- with fetal liver cells from β_2m normal mice, led to an iron shift from hepatocytes to Kupffer cells within one month, followed by a decrease in total iron stores at three months after reconstitution. However, during this period, transferrin saturation and mucosal transfer remained high. Hence, the histopathological analysis indicated an involvement of haematopoietic-derived cells, Kupffer cells or others, in regulating cellular iron storage in the liver (Santos et al., 1996). The importance of the lymphocytes as "protectors" of iron-induced toxicity was strengthened by a recent study demonstrating that the severity of iron overload was considerably enhanced when the RAG-1 mutation (which causes a lack of mature lymphocytes) was introduced into β_2m -/- mice (Santos et al., 1999). Moreover, β_2m -/- RAG-1 -/- mice developed heart fibrosis, which was prevented by reconstitution with normal haematopoietic cells (Santos et al., 1999). Finally, a recent study with TCRγδ -/- mice has suggested that these cells may contribute to the gut's TNF-α response to iron, which would affect the iron deposition within the liver (Ten-Elshof et al., 1999).

The molecules and possible mechanisms

Several genes involved in iron metabolism and in the regulation of oxidative stress are modulated by soluble factors such as cytokines and nitric oxide (NO). Thus, cells of the immune system such as lymphocytes and macrophages by releasing these soluble factors may regulate cellular iron metabolism and iron-induced toxicity.

Regulation of cellular iron metabolism

Cytokines have been implicated in modulating synthesis of several proteins involved in iron metabolism, and thus cellular iron handling. Their effects and producing cells are summarised in Tables 1.1 and 1.2, respectively.

TNF-α was shown to induce H-ferritin in human myoblasts by transcriptional mechanisms, independently of iron and ferritin levels (Miller et al., 1991). In the human hepatoma cell line, HepG2, the cytokines TNF-α, IL-1β and IL-6 have been shown to induce Fe-transferrin uptake (Table 1.1). The observed increase in the biosynthesis of H-ferritin may contribute to efficient storage of acquired iron (Hirayama et al., 1993). Another study showed that IL-6 increased hepatic ferritin content, and Fe-transferrin uptake in isolated rat hepatocytes, while in Kupffer cells no significant increase in Fe-tranferrin uptake was observed (Table 1.1; Kobune et al., 1994). *In vivo* administration of TNF-α, resulted in a significant decrease in total red blood cell (Moldawer et al., 1989). Thus, these data indicate that inflammation-related cytokines promote iron uptake and storage by the hepatocytes, contributing to hypoferraemia (Weiss et al., 1995).

Interferon-γ (IFN-γ) leads to decreased uptake of Fe-Tf in cells from the reticuloendotelial system (Table 1.1). However, several differences in the expression of proteins involved in iron handling such as ferritin and transferrin receptor, were observed in those studies, which may be dependent on the cell type analysed and the time of treatment with this cytokine. In addition other cytokines such as IL-4, IL-13 and IL-2 were also reported to modulate the expression of such proteins (Weiss et al., 1997; Seiser et al., 1993).

Table 1.1 Documented influences of cytokines in iron metabolism.

Target cells	Cytokine	Influence			References
		Protein		Fe-Tf uptake	
		TfR	Ferritin		
HepG2, human heptoma cell line	TNF-α	↑	↑	↑	Hirayama et al., 1993
	IL-1β	↑	↑	↑	Hirayama et al., 1993
	IL-6	↑	↑	↑	Hirayama et al., 1993
Rat hepatocytes	IL-6	ND	↑	↑	Kobune et al., 1994
U937, human monocytic cell line	IFN-γ	↔	↑ (H); ↔ (L)	↓	Fahmy and Young, 1993
	TNF-α	↓	↑ (H); ↔ (L)	↓	Fahmy and Young, 1993
	IL-1β	↓	↔ (H and L)	↓	Fahmy and Young, 1993
Human monocytes	IFN-γ	↓	↓	↓	Byrd and Horwitz, 1989; 1993
Human macrophages	IFN-γ	↑	↓	↓	Taetle and Honeysett, 1988
Rat Kupffer cells	IL-6	ND	ND	↔	Kobune et al., 1994

Abbreviations: ND, not determined, Tf, transferrin; TfR, transferrin receptor.

Several cytokines including TNF-α, IL-1β, IL-6 (Ramadori et al., 1988) and IFN-γ (Mazumder et al., 1997) induce ceruloplasmin synthesis by hepatoma cell lines. Induction of ceruloplasmin by IFN-γ was also observed in human monocytic cells (Mazumder et al., 1997). Ceruloplasmin is a copper oxidase implicated in iron metabolism because of its catalytic oxidation of Fe^{2+} to Fe^{3+} (ferroxidase activity) (Curzon and O'Reilly, 1960). Premature termination mutations in the ceruloplasmin gene, lead to a total absence of ceruloplasmin (aceruloplasminaemia) which is associated with a severe haemosiderosis (Yoshida et al., 1995). Despite the clinical description of aceruloplasminaemia, the role of ceruloplasmin in iron homeostasis has been confusing. Early studies, in copper-deficient animals revealed impaired reticuloendothelial cell iron release, that was corrected by the administration of ceruloplasmin, implying a role for this protein as a plasma ferroxidase regulating tissue iron efflux

Table 1.2 Cells known to synthesise and secrete cytokines relevant to iron metabolism*.

Cytokine	Producing cell(s)**
IL-1α, IL-1β	macrophages, fibroblasts, keratinocytes of the skin, T and B cells, astrocytes, microglial cells
IL-6	macrophages, T cells
IFN-α, IFN-γ	T cells
TNF-α	activated macrophages

* The effects of these cytokines in iron metabolism are summarised in Table 1.1.

** Reviewed by Arai et al., 1990.

(Lee et al., 1968; Roeser et al., 1970; Osaki et al., 1971). In addition, iron release from HepG2 hepatoma cell line was accelerated by ceruloplasmin, specially when iron release was occurring rapidly (stimulated by apotransferrin), or under conditions of limited oxygen (Young et al., 1997). However, later work with cultured cell lines *in vitro* has suggested a role for ceruloplasmin in cellular iron influx (Mukhopadhyay et al., 1998; Attieh et al., 1999). This effect was observed in iron-deficient cells. In a recent study, an essential role for ceruloplasmin in determining the rate of iron efflux from cells with mobilisable iron stores was observed in the ceruloplasmin knockout mice (Harris et al., 1999). This impairment in the movement of iron out was observed both in reticuloendothelial cells and hepatocytes. Thus, although ceruloplasmin may have a role in iron uptake under certain iron-deficient conditions, it is more likely that it plays an essential role in iron efflux. In this scenario, cells from the immune system by releasing cytokines that up-regulate the expression of ceruloplasmin, may decrease intracellular iron content, specially under conditions of iron overload.

The *Nramp1* gene (Govoni et al., 1997), which encodes a protein that has homology with the divalent metal transporter (DMT1), is another gene that is regulated by IFN-γ. Nramp1 protein is exclusively expressed in the endosomal compartment of monocytes and macrophages and appears to mobilise iron from the intracellular lysosomes (Atkinson and Barton, 1998; Gomes and Appelberg, 1998).

Activation of macrophages with IFN-γ and LPS, induce NO synthase activity, with consequent increase of the levels of NO production (Nathan, 1992; Moncada and Higgs, 1993). NO regulates iron homeostasis, mainly by modulating the activity of the iron regulatory proteins (IRPs) (Drapier et al., 1993; Hentze and Kuhn, 1996; Domachowske, 1997; Oliveira et al., 1999). IRP1 and IRP2 are two key proteins of iron metabolism that regulate ferritin and transferrin receptor expression upon binding to the iron regulatory elements (IRE) located in the 5' and 3' untranslated regions of their messengers RNAs (mRNAs), respectively. This binding prevents translation of ferritin mRNA and stabilises transferrin receptor mRNA (Hentze and Kuhn, 1996). The effects of NO on IRPs are different, depending on the cell type analysed (Richardson et al., 1995; Oria et al., 1995; Cairo and Pietrangelo, 1995; Phillips et al., 1996; Sanchez et al., 1996; Recalcati et al., 1998; Barisani et al., 1999). In summary, the role of NO in iron metabolism is complex and may involve different pathways.

Regulation of oxidative stress and fibrosis

As mentioned above, increased tissue iron concentrations can promote oxidative injury via iron-catalysed reactive oxygen species (Ryan and Aust, 1992). Ferritin, the major intracellular iron-binding complex, protecting the cell from the damaging actions of iron, can be induced by

TNF-α and IL-1β. Thus, these cytokines might have a protective part, by decreasing the intracellular free iron. However, in the human lung epithelium-like adenocarcinoma cell line A549, treated with TNF-α and IL-1β, the enhanced synthesis of H-ferritin was not fully protective against iron toxicity because these cytokines enhanced, concomitantly, the uptake of non-transferrin-bound iron, which in turn was associated with peroxidation of cell lipids and increased cell death (Smirnov et al., 1999). Hence, the role for cytokines in regulating iron handling seems to be a complex process. Ceruloplasmin, whose expression is up-regulated by IFN-γ as referred above, was shown to inhibit superoxide-dependent mobilisation of iron from ferritin, suggesting that ceruloplasmin represses lipid peroxidation by decreasing the availability of iron from ferritin (Samokyszyn et al., 1991). In other studies, TNF-α and IL-lα induced oxidant-protective mechanisms such as superoxide dismutases (Visner et al., 1991), metallothionein (Kaji et al., 1993) and haem oxygenase-1 expression in endothelial cells (Terry et al., 1998). In addition, NO also modulates the activity of haem oxygenase leading to a major resistance of the endothelium to oxidative stress (Motterlini et al., 1996). NO has been shown to act as an antioxidant in iron-mediated oxidative stress in rat hepatocytes, by reacting with a low molecular weight complex of iron (Kanner et al., 1991; Sergent et al., 1997). Antioxidant effects of NO result also from its direct reaction with alkoxyl and peroxyl radical intermediates during lipid peroxidation, thus terminating lipid radical chain propagation reactions (Rubbo et al., 1994). In spite of NO mediating tissue protection during oxidative stress, it can be toxic as well by exhibiting pro-oxidant effects. Relative rates of production and steady state concentrations of NO, and the cellular sites of production seem to influence profoundly the expression of the differential oxidant injury-enhancing and protective effects of NO (Rubbo et al., 1996; Rubbo, 1998; Li and Billiar, 1999).

In humans with iron overload due to either genetic or acquired causes, fibrosis and cirrhosis are hallmarks of liver disease. However, the pathological mechanisms underlying the fibrinogenic potential of iron remain poorly understood. Hepatic fibrosis is a dynamic process, bridging the pathway from chronic liver damage to cirrhosis. It is predominantly characterised by excessive accumulation of extracellular matrix (ECM) components in the liver, caused by its markedly increased production and unbalanced degradation. Evidence for altered hepatic matrix degradation has been shown in HH (George et al., 1998).

Currently, hepatic stellate cell activation during fibrogenesis is seen as a dynamic stepwise process, in which morphological and biochemical changes lead to a metabolically active "myofibrolast-like" cell from a quiescent one: in normal liver, stellate cells show a resting phenotype and are mainly involved in vitamin A storage (Hendriks et al., 1987), while during chronic liver disease, these cells undergo phenotypic differentiation, proliferate and synthesise

various ECM components, such as collagen (Friedman, 1993). In an animal model of HH, activation of the collagen type I gene was observed in stellate cells (Pietrangelo et al., 1995). In other studies, increased amounts in collagen deposition, which occurred following a transient increase in transferrin saturation *in vivo* after an intravenous injection of ferric citrate, was associated with concomitant increase in numbers of T lymphocytes in the synovium (de Sousa et al., 1990). Interestingly, potent antifibrogenic effects of IFN-γ, and also of IFN-α in some cases, have been documented in several cells of mesenchymal origin, including scleroderma fibroblasts (Amento et al., 1985; Goldring et al., 1986; Rosenbloom et al., 1986; Granstein et al., 1987; Nanes et al., 1989; Reginato et al., 1993). Importantly, IFN-γ, and also IFN-α were demonstrated to inhibit proliferation and collagen synthesis of cultured human myofibroblastic stellate cells (Mallat et al., 1995; Tiggelman et al., 1995).

As mentioned above, fibrosis is the net result of an imbalance between synthesis and degradation of ECM components, mainly collagen. In this context, accumulated evidence indicates that altered matrix degradation is implicated in liver injury and fibrosis (Arthur, 1994; Iredale et al., 1998). Degradation of ECM proteins is regulated by a family of enzymes called the matrix metalloproteinases (Matrisian, 1990). Collagenase gene expression is modulated by cytokines such as TNF-α and IL-1α (Tamura et al., 1996; Borden and Heller, 1997; Burger et al., 1998). Despite growing evidence for altered matrix turnover in liver fibrosis, the role of this matrix-degradating system in normal and diseased liver is not clearly defined yet (Pietrangelo, 1996). Thus, although some cytokines are currently seen as inducers of fibrosis (Gressner, 1996), the same may also exhibit anti-fibrotic properties.

AIMS OF THIS THESIS

As outline above the immune system may play a role in regulating iron homeostasis and iron-induced toxicity. The work carried out previously was mainly done in lymphocytes from peripheral blood of HH patients, and in animal models. Whether, in HH patients, there was an abnormal lymphocyte distribution or defective function of lymphocytes in affected tissues such as liver, remained unknown. Thus, the general aim of this thesis has been to try to elucidate immune-mediators underlying "protection" in conditions of iron overload, by analysing *in vivo* the lymphocyte subpopulations in the liver tissue, in models of iron accumulation and relate these observations with hepatic toxicity of iron.

The specific aims were:

- to characterise the group of HH patients studied with regard to the genotype of the *HFE* gene, discovered when this study had already started (chapter 2).
- to investigate whether low numbers of $CD8^+$ cells in peripheral blood of HH patients were associated with low numbers in the liver (chapter 3).
- to examine whether the number of $CD8^+$ cells in the liver of HH patients were related to the clinical expression of the disease measured by iron stores and liver damage (chapter 3).
- to define further which hepatic lymphocyte subsets may be involved in regulating iron-induced toxicity in the liver, in an animal model of iron overload (chapter 4).

CHAPTER 2 - *HFE* MUTATIONS IN HH PATIENTS IN SWEDEN

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INTRODUCTION

Hereditary haemochromatosis (HH) is an autosomal recessive disease, affecting approximately 1 in 200 to 1 in 400 Caucasian individuals (reviewed by Merryweather-Clarke et al., 1997), which is characterised by gradual iron overload within parenchymal cells of several organs, principally liver, pancreas, and heart, leading, if left untreated, to clinical manifestations in early adulthood such as liver cirrhosis, hepatocellular carcinoma, diabetes and chronic heart (Powell et al., 1994). In 1996 the gene for HH was cloned by Feder et al. The gene is located on chromosome 6, three megabases telomeric of the HLA region, and is the latest member of the major histocompatibility complex (MHC) class Ib gene family. This gene, first described as *HLA-H*, was renamed *HFE*, following several studies have confirmed that this gene was responsible for the vast majority of HH Caucasian patients. 64 %-100 % of HH patients are homozygous for a single mutation, C282Y (Feder et al., 1996; Beutler et al., 1996; Calandro et al., 1996; Jazwinska et al., 1996; Jouanolle et al., 1996; Carella et al., 1997; Piperno et al., 1998; Bacon et al., 1999). A second mutation, resulting in a histidine-aspartate substitution at position 63 (H63D), was reported to be increased in patients with HH heterozygous for the C282Y mutation (Feder et al., 1996; Beutler et al., 1996). To characterise the genotype of the group of Swedish HH patients studied in the present investigation, the presence of these two mutations was evaluated.

SUBJECTS AND METHODS

Subjects

Eighty-seven HH Swedish patients, 67 men with a mean age of 47 years (20-73), and 20 women with a mean age of 49 years (25-72) were examined. Diagnostic criteria used were high transferrin saturation (> 60 % in males and > 50 % in females) and increased ferritin levels > 300 μgL^{-1} (except in one 20 years old patient) and a liver biopsy with typical iron staining indicating primary haemochromatosis according to Scheuer et al. (1962). All patients included in the study were from a clinical, biochemical and histopathological point of view homozygous for HH (Powell et al., 1994). Siblings to patients with HH in the study were excluded. Patients were treated weekly with phlebotomies of 450 ml (1 L of blood contains approximately 0.5 g of iron). Patients with other causes for iron overload, such as excessive intake of oral supplementation, were excluded and none had received more than two units of blood from transfusions. Alcohol consumption was

less than 30 g per day in all patients except one, who consumed 60 g of alcohol per day. All patients were negative for antibodies to hepatitis C, tested with recombinant immunoblot assay II (Chiron Corporation, Emeryville, CA). Of the male patients with 5 g or less (n = 11) one was blood donor, one had ulcerative colitis, otherwise we have no explanation as to why they have so little iron. DNA from 117 random healthy Swedish subjects was used as controls. The individual identity of these samples is not known.

Methods

Genomic DNA was isolated from blood using QIAamp Blood Kit (QIAGEN, Germany). Two portions of the *HFE* gene surrounding both mutations were amplified separately by the polymerase chain reaction (PCR) using *HFE* specific primers (for the C282Y mutation, forward primer: 5'- GTG ACC TCT TCA GTG ACC, and reverse primer: 5'- AAT GAG GGG CTG ATC CAG; for the H63D mutation, forward primer: 5'-ATG GGT GCC TCA GAG CAG, and reverse primer: 5'- AGT CCA GAA GTC AAC AGT). PCR was performed using GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT) in a final volume of 100 µl. The PCR mixture contained 1× buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 mM of each dNTP, 0.3 µM of each primer, 2.5U of Taq DNA polymerase (Boehringer Mannheim, Germany) and 80-100 ng of DNA. The PCR was carried out for 35 cycles (annealing at 62 °C and 50 °C for the C282Y and H63D mutation, respectively). Ten µl of the amplified products were digested with *Sna* BI (BioLabs, New England, MA, USA) for the C282Y mutation or with *Bcl* I (BioLabs) for the H63D mutation according to the manufacturer's recommendations. The digested products were then run on a 4% NuSieve 3:1 agarose (FMC BioProducts - Europe, Denmark) gel at 100 V for 1.5 h and photographed under UV light. The C282Y mutation creates a *Sna* B I restriction site. Thus, the 237-bp PCR reaction product digested with *Sna* B I shows two fragments of 197 and 40-bp in mutant DNA. In normal DNA, no *Sna* B I site is created and, therefore, only one fragment corresponding to the 237-bp PCR product is visible in the gel (Figure 2.1a). The H63D mutation removes the *Bcl* I site in the 210-bp PCR product, while in normal DNA *Bcl* I digestion results in two fragments of 129 and 81-bp (Figure 2.1b).

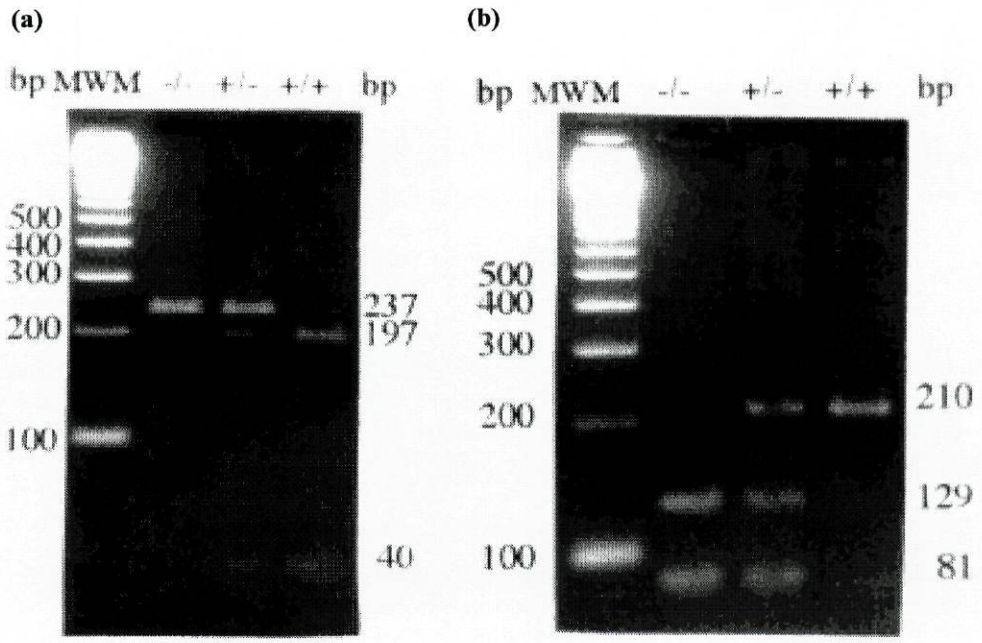


Figure 2.1 NuSieve gel electrophoresis of the PCR fragments digested by enzyme restriction. (a) diagnosis of the C282Y mutation; (b) diagnosis of the H63D mutation. Band sizes (in bp) are shown on the lateral sides; the molecular weight marker (MWM), is the 100 bp ladder (Pharmacia Biotech, Sweden).

Statistical analyses

Statistical significance of the data was determined by the χ^2-test. To study the linear relationship between age and iron stores, the Pearson's correlation coefficient was estimated. A *P*-value of < 0.05 was considered significant.

RESULTS

The *HFE* genotypes for HH patients and normal subjects are summarised in Table 2.1. Of the patients, 92.0 % were homozygous and 4.5 % were heterozygous for the C282Y mutation. Three HH patients were negative for this mutation. No homozygous for the C282Y mutation was found amongst the control subjects.

Table 2.1 Analysis of mutations in the *HFE* gene in 87 patients with hereditary haemochromatosis and 117 normal subjects

Genotypes		Patients*	Controls*
C282Y	**H63D**	n=87	n=117
+/+	-/-	80 (92.0)	0
+/+	+/-	0	0
+/+	+/+	0	0
+/-	-/-	1 (1.1)	9 (7.7)
+/-	+/-	3 (3.4)	0
+/-	+/+	0	0
-/-	-/-	1 (1.1)	80 (68.4)
-/-	+/-	1 (1.1)	27 (23.1)
-/-	+/+	1 (1.1)	1 (0.8)

* Number of subjects with the genotype. Parentheses denote percentage.

As can be seen in the Figure 2.2 there was no correlation between iron stores and age at diagnosis, even when men and women were analysed separately ($r = 0.22$, $n = 61$, $P > 0.05$; $r = -0.13$, $n = 19$, $P > 0.05$, respectively). Although heterozygous patients had low iron stores, ten patients over 39 years old who were homozygous for the C282Y mutation had removed 5 g or less of iron. Of these, one patient was a blood donor and one had ulcerative colitis.

As shown in the Table 2.2, the C282Y mutation frequency was higher in HH patients than in control subjects. The frequency of H63D was higher in the controls than in the patients with HH. However, because the C282Y mutation is in complete linkage disequilibrium with the H63D mutation, the only chromosomes at risk for the H63D mutation are those without the C282Y mutation. Thus, six of the 10 chromosomes at risk carried the H63D mutation, giving a frequency of 60 % in HH patients as compared to the controls where 29 of 225 chromosomes at risk carried the H63D mutation giving a frequency of 13 % ($P < 0.001$).

Table 2.2 Mutation frequencies in the *HFE* gene in 87 patients with hereditary haemochromatosis and in 117 normal subjects

Mutation	Patients* n=174	Controls* n=234	*P***
C282Y	164 (94.2)	9 (3.8)	< 0.001
H63D	6 (3.4)	29 (12.4)	< 0.01

* Number of chromosomes with mutation. Parentheses denote percentage

**χ^2-test.

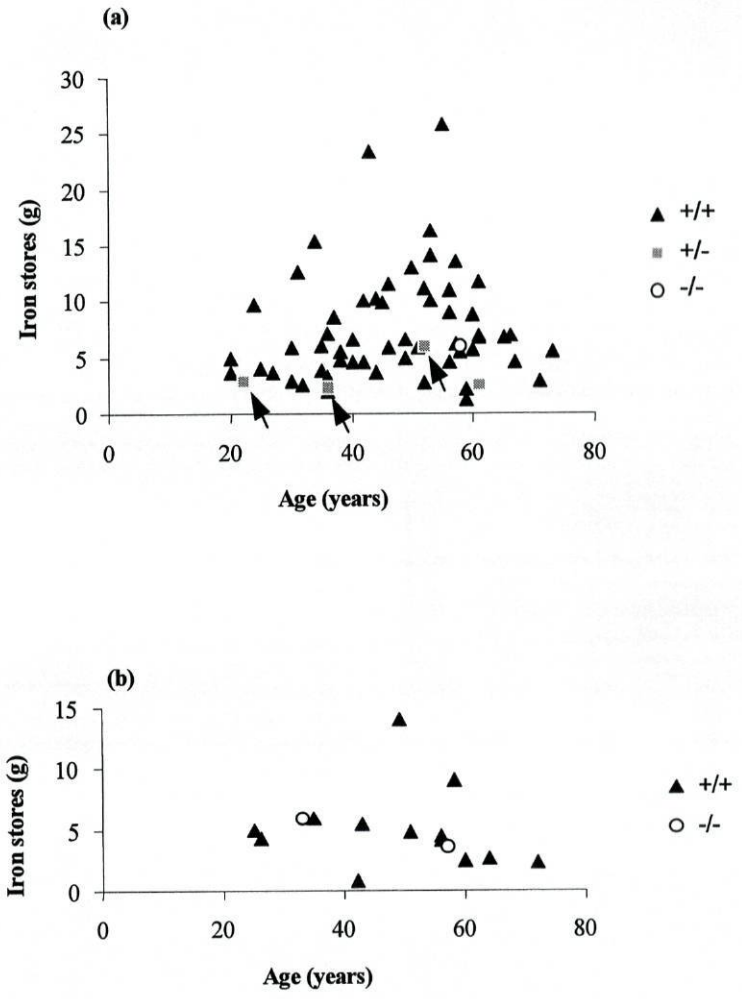


Figure 2.2 Relationship between iron removed by phlebotomies and age at diagnosis in males (a) and in females (b) with hereditary haemochromatosis. Symbols in open circles denote patients negative for the C282Y mutation, in squares heterozygous, and in triangles homozygous for this mutation. Patients in whom phlebotomy treatment had not been terminated are not represented. The arrows indicate heterozygousity for both C282Y and H63D mutations.

Table 2.3 Clinical data on three out of 87 patients with hereditary haemochromatosis who did not carry the C282Y mutation

Patient no.	Age/ Sex	Genotypes		ALT* (μkatalL^{-1})	Serum iron* (μmolml^{-1})	Serum transferrin saturation* (%)	Serum ferritin* (μgL^{-1})	Liver biopsy iron grade§	Removed iron (g)	Liver histology	Related symptoms/ relatives
		C282Y	H63D								
1	33/F	-/-	-/-	0.36	40	89	804	3	6.0	Fibrosis	Arthritis
2	57/F	-/-	+/-	1.20	47	89	746	3	3.6	Fibrosis	Arthritis/ sibling with HH
3	58/M	-/-	+/+	1.26	44	93	1410	3	6.1	Fibrosis	Diabetes

*Normal values: alanine transaminase (ALT): males < 0.8 μkatalL^{-1}, females < 0.6 μkatalL^{-1}; serum iron: 11-32 μmolml^{-1}; Serum transferrin saturation: 10-60%; serum ferritin: 9-210 μgL^{-1}. §Hepatic iron overload grading according to Scheuer et al., 1962.

The clinical data of patients with normal alleles for the C282Y are presented in Table 2.3. As can be seen they all showed many features typical of haemochromatosis, both biochemical and clinical.

DISCUSSION

Two main mutations in the *HFE* gene have been described (Feder et al., 1996). The C282Y variant disrupts the interaction of the *HFE* chain with the β_2-microglobulin and thereby eliminates cell-surface presentation (Feder et al., 1997). The other mutation (H63D) does not interfere with the expression of the HFE protein on the cell surface. The role of the H63D mutation in HH is still not completely clear.

We found the C282Y mutation in approximately 94 % of chromosomes from HH patients, while only approximately 4 % of chromosomes from the control population carried this mutation. It has been shown that the C282Y mutation of the *HFE* gene is present in a high percentage of Caucasian HH patients. However, the frequency varies between groups from different countries (Feder et al., 1996; Beutler et al., 1996; Calandro et al., 1996; Jazwinska et al., 1996; Jouanolle et al., 1996; Carella et al., 1997; Piperno et al., 1998). In Australia, the C282Y mutation was present in 100 % of HH patients (Jazwinska et al., 1996). In contrast, only 64-69 % of chromosomes from Italian patients with HH carried this mutation (Carella et al., 1997; Piperno et al., 1998). Our study confirms that the C282Y mutation is strongly associated with HH. However, despite most of the patients having the C282Y mutation, they presented very different levels of iron stores, which was not related with the age of the patients at diagnosis, which is in agreement with other studies (Porto et al., 1994; Adams et al., 1997). Thus, the discover of the *HFE* gene does not seem to be sufficient to explain the clinical heterogeneity of HH.

One of the C282Y negative HH patients was homozygous for the H63D mutation. However, the importance of this homozygousity is not known, since one of the control subjects was also homozygous for this mutation. All patients negative for the C282Y mutation (Table 2.3) had several clinical findings strongly indicating the diagnosis of HH, such as high transferrin saturation, associated symptoms, and in one patient, affected relatives. The fact that there are patients who do not have any of these two mutations suggests that additional mutations may be present either within the HLA region or on other not yet detected loci. Accordingly, evidence for multiple genes linked to the MHC being responsible for haemochromatosis has been suggested (Barton et al., 1997), as well as the occurrence of HH in patients without mutations in the *HFE* gene, who revealed no linkage of the haemochromatosis phenotype with the short arm of chromosome 6, the site of *HFE* gene (Pietrangelo et al., 1999). In the present study, we found a similar frequency of the *HFE* C282Y mutation in Sweden, as compared with data from USA, Australia and France, but a considerably higher frequency than that reported from Italy (Carella et al., 1997).

The role of the other mutation (H63D) in the phenotypic expression of HH is unclear. In controls, the frequency of H63D mutation is higher than the C282Y mutation. If the H63D mutation had a critical role in the development of HH, we would expect to find more homozygous patients for this mutation. However, amongst four HH patients heterozygous for the C282Y mutation three were also heterozygous for the H63D mutation, compared to the controls where none of the individuals heterozygous for the C282Y mutation had the H63D mutation. This indicates that either this mutation is in linkage disequilibrium with other yet undiscovered mutations, or that the H63D itself is a deleterious mutation, which together with the C282Y mutation causes haemochromatosis. Since we found that of the 10 chromosomes at risk in the patients, 6 (60%) had the H63D mutation, this mutation may be important for the expression of the HH.

The frequency of HH differs among different countries and even within the same country (Porto and de Sousa, 2000). Sweden has also shown variations in the prevalence of HH (Olsson et al., 1978; 1983; 1984; 1996; Lindmark and Eriksson 1985; Hallberg et al., 1989). In healthy controls, no homozygous person for the C282Y mutation was found, while 7.7% were heterozygous, giving an allelic frequency of 3.8% for the C282Y mutation. If this mutation was indeed alone responsible for HH, the prevalence for HH would be 0.15%. This corresponds well with previously published frequencies on the prevalence of HH in the Stockholm area (Hallberg et al., 1989).

CHAPTER 3 – HEPATIC DAMAGE IN C282Y HOMOZYGOTES RELATES TO LOW NUMBERS OF $CD8^+$ CELLS IN THE LIVER LOBULI

(Modified from: European Journal of Clinical Investigation, in press)

INTRODUCTION

Although most Caucasian patients with HH show the same mutation (C282Y) in the *HFE* gene, the phenotypic expression of the disease varies greatly. Furthermore, it was reported that there are asymptomatic homozygous C282Y subjects (Adam et al., 1997; Rhodes et al., 1997) and that there are affected HH patients who do not have any mutation in the *HFE* gene (Pietrangelo et al., 1999). Thus, the discovery of the *HFE* gene is far from being sufficient to explain or predict the variation in the expression of iron overload.

Studies of peripheral blood cells from patients with HH have shown that defective numbers of some T-lymphocyte populations contribute to the phenotypic heterogeneity in HH (reviewed by de Sousa and Porto, 1998). Patients with low numbers of peripheral blood $CD8^+$ T-lymphocytes have a more severe iron overload (Reimao et al., 1991; Porto et al., 1994). It was also shown that each individual's $CD4^+/CD8^+$ ratio, as well as absolute $CD4^+$ and $CD8^+$ cell numbers, remain unaffected after treatment, confirming a strict homeostatic regulation of the relative numbers of the two major peripheral T-lymphocyte populations (Reimao et al., 1991; Porto et al., 1994; Porto et al., 1997).

A pattern of iron overload accumulation similar to that found in HH has been described in the *HFE* knockout mice (Zhou et al., 1998) and earlier in β_2-microglobulin knockout mice (de Sousa et al., 1994; Rothenberg et al., 1996; Santos et al., 1996). Moreover, the severity of iron overload pathology is considerably enhanced when a RAG-1 mutation (which causes a lack of mature lymphocytes) is introduced into *β_2m-/-* mice (Santos et al., 1999). A recent study with *TCR$\gamma\delta$-/-* mice has suggested that these cells may contribute to the gut's TNF-α response to iron, which would affect the iron deposition within the liver (Ten-Elshof et al., 1999). Thus, strong evidence points towards MHC-class I molecules and lymphocytes being involved in iron homeostasis (de Sousa and Porto, 1998).

In humans, studies of lymphocyte numbers in HH have been confined to the blood compartment, although $CD3^+$ cells have been observed but not quantified in the liver of HH patients (Stal et al., 1995). It is known that some subsets of lymphocytes show preferential migration to particular tissues including the liver (Khakoo et al., 1997; Nuti et al., 1998). Therefore, further characterisation of $CD8^+$ cells in the liver, is important as a first step for clarifying their putative role under deregulated iron absorption leading to iron overload. To better evaluate the pattern of $CD8^+$ cell distribution in different tissues in HH, cells were quantified in liver and small intestine sections, and peripheral blood samples. All HH patients studied were homozygous for the C282Y mutation and lymphocyte numbers were examined. In order to

investigate one possible mechanism underlying the role of $CD8^+$ cells in iron overload the expression of TIA-1 molecule, which is a granule-associated protein expressed by cytotoxic cells was also analysed (Anderson et al., 1990; Tian et al., 1991).

PATIENTS AND METHODS

Patients

Thirty-eight patients (31 men and 7 women, aged 20 to 73 years) with untreated HH were investigated. The diagnosis was based on high serum iron (greater than 30 μM), transferrin saturation greater than 60% in men and 50% in women, serum ferritin levels higher than 300 μg/L and liver biopsy with typical iron staining by the Perls' method, indicating primary haemochromatosis (Scheuer et al., 1962). Intensive treatment was completed when the serum ferritin was less then 10 μg/ml, often in combination with anaemia. Liver studies were performed in 37 HH patients. Six HH patients (no. 14, 16, 20, 26, 27 and 35) had cirrhosis and seven (no. 10, 11, 18, 21, 24, 25 and 37) had fibrosis. Except for the positive Perls' iron staining in hepatocytes, the remaining 25 liver sections had normal histology (Table 3.1). Liver biopsies were repeated in six patients (no. 2, 10, 11, 21, 27 and 35) after the completed venesection treatment. After treatment, iron staining in the liver had Scheuer scoring equal to zero (Scheuer et al., 1962). All HH patients were homozygous for the C282Y mutation in the *HFE* gene (Cardoso et al., 1998 and chapter 2). Exclusion criteria included chronic hepatitis (Desmet et al., 1994) or alcohol consumption greater than 50 g of alcohol per day. None of the patients had a history of oral iron supplementation, blood transfusion or had served as a blood donor.

Patients were treated with weekly phlebotomies removing 450 ml of blood (each 500 ml of blood contains approximately 250 mg iron). Body iron stores were calculated from the amount of blood removed during phlebotomies (Pietrangelo et al., 1999).

As controls, six subjects who had undergone liver biopsy because of slightly increased alanine transaminase (ALT) values, and three subjects treated with methotrexate who were biopsied in order to evaluate the risk for fibrosis development due too the drug, were used. None of them had iron overload or chronic liver disease and, except for slight fatty infiltration, all had normal liver histology. Liver biopsies from 11 patients with cirrhosis due to alcoholic liver disease (ALD, 6 patients) or hepatitis C virus (HCV, 5 patients) served as an additional control group. All

these patients and the normal controls had Scheuer scoring equal to zero (Scheuer et al., 1962) and were not genotyped for *HFE* gene.

Biopsies from the small intestine were obtained from jejunum, just distal to the ligament of Treitz with a Crosby capsule. The tissue was fixed in buffered formalin and embedded in paraffin. The small intestine (SI) was investigated in 11 treated HH patients, one of which was not included in the liver studies, and 17 controls. Controls were subjects undergoing investigation for suspected celiac disease, but found to have a normal intestine morphology. The SI histology in all the HH patients was also normal.

All patients gave their informed consent to the investigation and the Ethical Committee of the Karolinska Hospital had granted approval for the study.

Histology

All biopsies were analysed by routine histological stainings, such as haematoxylin and eosin. Liver tissue was also processed for sirius staining to detect the presence of fibrosis, and Perls' staining for the presence of iron.

Immunohistochemistry

Six-micrometer formalin-fixed paraffin-embedded sections from liver or jejunum were mounted on Superfrost Plus glass slides (Menzel Glaser, Germany) dried overnight and incubated in an oven at 37 °C for 1 h. The sections were deparaffinised with xylene, and passed through graded alcohols. Sections were treated for epitope retrieval by incubation in a microwave oven 10 min in EDTA 1 mM, pH8 (750 W) and put at room temperature for about 30 min. Sections were stained using alkaline phosphatase-anti-alkaline phosphatase (APAAP), avidin-biotin-complex horse peroxidase (ABC-HRP) techniques as described below. Before performing the ABC-HRP method, endogenous peroxidase and endogenous biotin were blocked by incubating the sections with 0.3 % H_2O_2 for 30 min in the dark, and by using the Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA), respectively. An irrelevant mouse monoclonal antibody (mAb) (clone DAK-GO1, DAKO, Glostrup, Denmark) was used as a negative control in a 1:100 dilution. The antibody specificity of DAK-GO1 is directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues. Sections of palatine tonsils were used as positive controls and also to determine optimal antigen retrieval and optimal titers of the mAb used. Reproducibility of the lymphocyte counts was tested by examining liver sections

done at different levels of the paraffin blocks from the same subjects (no. 2, 10, 11, 21, 27, 35, Table 3.1). The results were highly reproducible (Table 3.1).

APAAP method

In the liver, immunohistochemical staining was performed by the APAAP method. To reduce non-specific binding, normal rabbit serum diluted 1:10 was applied for 10 min. Then, after blotting the excess of serum, sections were incubated overnight with purified mouse anti-CD8 mAb (clone C8/144B, DAKO) diluted 1:100. After washing in tris-buffered saline (TBS) the second-layer, rabbit anti-mouse immunoglobulin code Z259 (DAKO) was applied for 30 min and then, after washing again, the mouse APAAP conjugate code D651 (DAKO) was applied for 30 min. Incubation steps with the link antibody (rabbit anti-mouse, DAKO) and the APAAP conjugate (DAKO), were repeated for 10 min each, to increase the intensity of the reaction. After additional washings, colour was developed with Fast-Red substrate (DAKO). Sections were counterstained in Mayer's haematoxylin and mounted in KAISER's glycerol gelatine.

ABC-HRP method

In the small intestine immunohistochemical staining was performed using the avidin-biotin-complex method, since this tissue is rich in endogenous alkaline phosphatase. Briefly, sections were incubated for 20 min with normal goat serum diluted 1:10. Then, after blotting the excess of serum, sections were incubated overnight with purified mouse anti-CD8 mAb (clone C8/144B, DAKO) diluted 1:100, followed by incubations with biotinylated goat-anti-mouse IgG mAb (Caltag Laboratories, San Francisco, CA) diluted 1:300 for 30 min, ABC Elite complex (Vector Laboratories) for 30 min and diaminobenzidine as a chromogen (Vector Laboratories). Sections were counterstained in Mayer's haematoxylin and mounted in KAISER's glycerol gelatine. Each incubation was followed by washes in three changes of phosphate-buffered saline (PBS).

Double APAAP/TSA method

Double staining was performed with purified anti-CD8 mAb (clone C8/144B, DAKO) diluted 1:100 and anti-TIA-1 mAb (clone TIA-1, Coulter, Hialeah, FL), which was biotinylated using a commercial Kit (Boehringer Mannheim, Germany) and then used at a 1:1000 dilution. In the first part of the double staining, $CD8^+$ cells were labelled as described for liver sections, but without substrate addition. After incubation with APAAP, the substrate was not added since the substrate deposition would decrease the intracellular access of the second primary antibody. In the second part of the staining, sections were incubated with normal mouse serum diluted 1:5 (DAKO)

for 45 min and then with the biotinylated anti-TIA-1 mAb. The tyramide signal amplification method (NEN™ Life Science Products, MA) was used for demonstration of TIA-1, according to the manufacturer's instructions. Finally, the alkaline-phosphatase substrate, VECTOR Blue (Vector Laboratories) was added followed, after washing, by the peroxidase substrate, 3-amino-9-ethylcarbazole (AEC). In the double immunostaining, sections were not counterstained. Double APAAP/TSA staining was done in 15 liver biopsies from untreated HH patients and in 3 normal controls. The numbers of $CD8^+$ cells were similar by the APAAP/Vector Blue substrate, and the APAAP/Fast Red substrate methods. However, in the first one, in general, slightly fewer positive cells were seen. This could be due to the shorter incubation time done with the substrate in the double staining procedure in order to get a better visualisation of the two antigens. For each patient, when the double immunostaining was done, a single staining for each antigen was done in parallel. In these control slides the anti-TIA-1 or anti-CD8 mAbs, as well as the respective following reagents of the detection system, were substituted by the washing buffer. The total numbers of $CD8^+$ cells in the double stained slide were comparable to those obtained in the single stained slide. The total numbers of TIA-1^+ cells were slightly lower in the double stained slide in the majority of the patients. However, there was a very high correlation ($r = 0.88$) and a linear association between the counts in the double stained and control slides. Thus, results from the double staining were compared within the patients and controls. A liver biopsy from an HCV patient was used as an internal control between experiments.

Flow cytometry

Peripheral blood (PB) lymphocytes were analysed for T cell subsets by direct addition of labelled monoclonal antibodies (mAbs) to 50-100 µl of peripheral blood from treated HH patients. The following antibodies labelled with FITC or PE or PC-5 were used: anti-CD3-PC-5 (clone UCHT1), anti-CD4-PE (SFC12T4D11), anti-CD8-FITC (SFC121ThyD3), all from Coulter Corp., FL, with appropriate negative controls. After a 30-min incubation with mAbs, lysis and fixation were performed using Coulter Qprep (Coulter Corp.). The cells were then analysed by multiparameter flow cytometry using a Coulter Epics Elite (Coulter Corp.). The total leukocyte population was analysed, with gates for lymphocytes, monocytes, and granulocytes set according to their scatter properties and reactivity with anti-CD14-PE (Mo-2 for monocytes) and anti-CD45-FITC (KC56 for all leukocytes), with the lymphocytes identified as being $CD45^+/CD14^-$. The absolute number of lymphocytes was calculated from the percentage of lymphocytes (identified as described previously) and the total white blood cells count. The absolute number of cells in any

given lymphocyte subpopulation was calculated by multiplying the percentage of positive cells by the absolute number of lymphocytes.

Quantification of positive cells in the liver and small intestine

Quantification of positive cells was performed using a morphometric technique (Weibel and Elias, 1967). In short, all sections were examined "blind", i.e. without knowledge of their origin. In the liver, the number of $CD8^+$ cells was quantified separately in the lobuli and in the portal tracts. In case of extensive fibrosis or cirrhosis septa were considered as portal tract. Two observers examined the sections, although only one performed the counting. The quantification of positive cells was performed with a cross-hatched grid mounted into the ×10 eyepiece of the microscope (Nikon Eclipse E800). All positive cells wholly within the boundary of the grid and those intersected by or tangential to the upper and left grid edges were counted, while those intersected or tangential to the lower and right edges were excluded. Cell were counted, with a ×40 objective (corresponding to an area of 0.0625 mm^2 in the biopsy). In the lobuli the number of positive cells were counted in areas across the entire liver biopsy of each specimen (on average 50 fields were counted, minimum of 10 and a maximum of 143 fields); in each section the number of $CD8^+$ cells counted per field followed a distribution approximately normal, and the mean value was used for the calculations. In randomly selected patients, 2 serial sections were examined for control purposes, and a similar number of $CD8^+$ cells was observed ($r = 0.97$; $P<0.01$). Counts were expressed as the number of positive cells per mm^2.

The numbers of $CD8^+$ cells in the small intestine were counted at ×400 magnification and were expressed as the number of $CD8^+$ intraepithelial lymphocytes (IEL) per 100 epithelial cells and the number of $CD8^+$ lamina propria (LP) cells per 100 LP cells. Between 500-2000 epithelial cells and cells in the lamina propria were counted, depending on the size of the biopsy.

Statistical analysis

To study the linear relationship between continuous variables, Pearson's correlation coefficients were estimated. Differences between independent groups were tested by the nonparametric Mann-Whitney test. The nonparametric Wilcoxon test was used to compare the median of $CD8^+$ cells in liver biopsies before and after treatment. P-values < 0.05 were considered significant.

RESULTS

CD8$^+$ cells in the liver

The number of CD8$^+$ cells was quantified separately in the lobuli and in the portal tracts. The data presented refer to the results of counts of CD8$^+$ cells in the lobuli. A positive correlation was seen between the number of CD8$^+$ cells in the lobuli and in the portal tracts ($r = 0.52$; $P < 0.01$). CD8$^+$ cells were evenly distributed in the lobuli. However, striking differences in the number of CD8$^+$ cells between individual patients were observed (Figure 3.1A and 3.1B). Double staining for CD8 and TIA-1 showed that some but not all cells co-expressed the two molecules (Figure 3.1C).

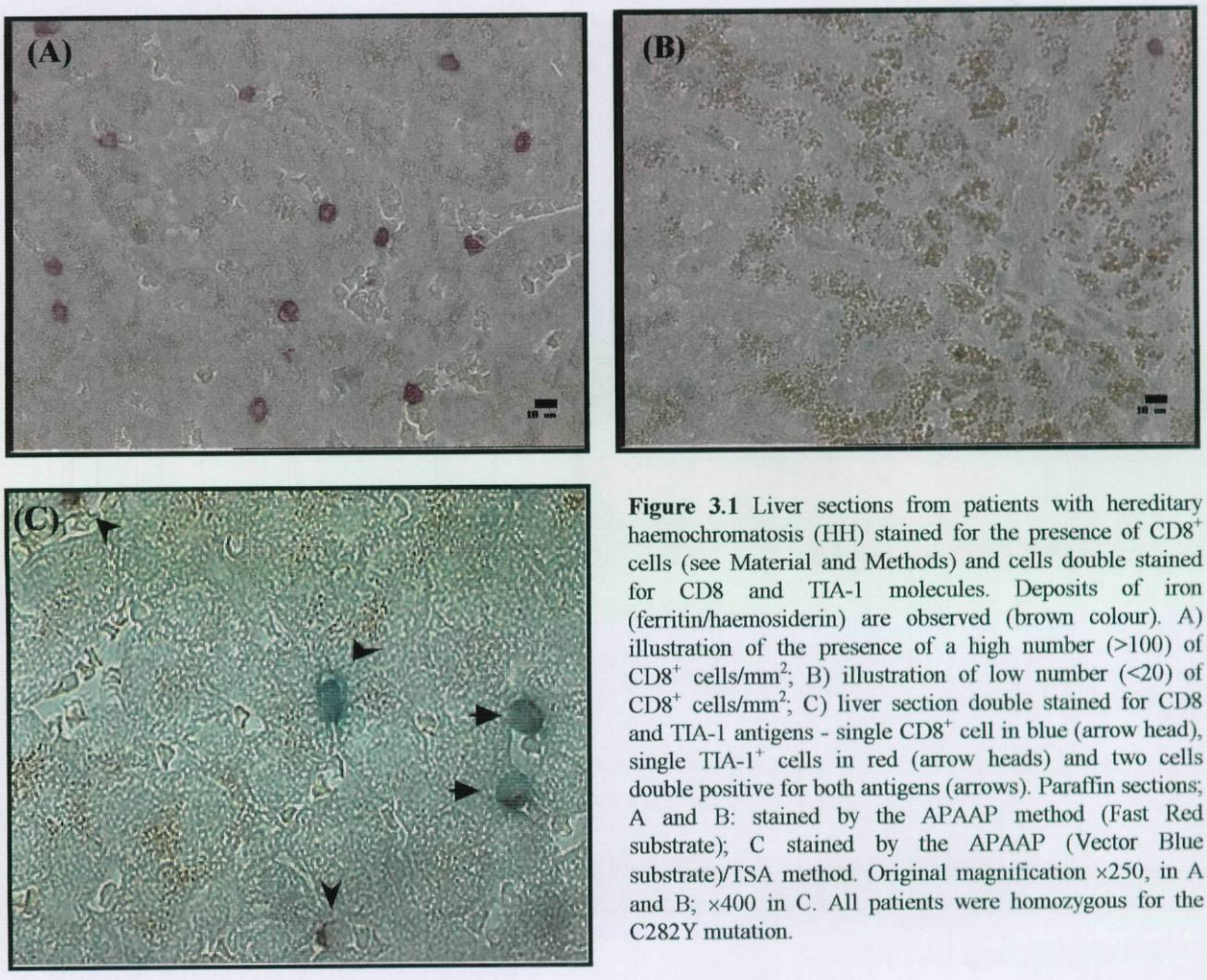


Figure 3.1 Liver sections from patients with hereditary haemochromatosis (HH) stained for the presence of CD8$^+$ cells (see Material and Methods) and cells double stained for CD8 and TIA-1 molecules. Deposits of iron (ferritin/haemosiderin) are observed (brown colour). A) illustration of the presence of a high number (>100) of CD8$^+$ cells/mm^2; B) illustration of low number (<20) of CD8$^+$ cells/mm^2; C) liver section double stained for CD8 and TIA-1 antigens - single CD8$^+$ cell in blue (arrow head), single TIA-1$^+$ cells in red (arrow heads) and two cells double positive for both antigens (arrows). Paraffin sections; A and B: stained by the APAAP method (Fast Red substrate); C stained by the APAAP (Vector Blue substrate)/TSA method. Original magnification ×250, in A and B; ×400 in C. All patients were homozygous for the C282Y mutation.

Table 3.1 Relevant data of HH patients studied before and after treatment with venesection therapy

Patient no.	Sex	Age (years)		Iron stores (g)	Fibrosis found on biopsy		No. $CD8^+$ cells in the lobuli/mm^2		$CD8^+TIA1^+$ (%)*
		BT	AT		BT	AT	BT	AT	BT
1	M	20		3.6	1		36		
2	M	24	25	9.7	1	1	37, 33	28	
3	F	25		5.0	1		44		
4	F	26		4.3	1		65		
5	M	27		3.6	1		36		
6	M	30		5.9	1		69		
7	M	32		2.5	1		87		8
8	M	32		8.4	1		58		
9	M	36		7.2	1		49		
10	M	37	38	8.6	2	2	31, 25	16	
11	M	40	41	4.5	2	2	61, 76	50	71
12	M	42		4.5	1		42		3
13	F	43		5.4	1		42		20
14	M	43		23.4	3		ND		
15	M	44		3.6	1		104		26
16	M	44		10.3	3		36		25
17	M	46		5.9	1		40		
18	M	46		11.5	2		34		
19	M	48		6.5	1		35		
20	F	49		14.0	3		12		
21	M	50	53	13.0	2	2	17, 17	58	56
22	M	51		5.9	1		24		0
23	M	52		2.7	1		34		9
24	M	52		3.4	2		26		
25	M	53		5.2	2		81		
26	M	53		14.1	3		19		
27	M	53	56	16.2	3	3	7, 6	26	
28	M	55		7.9	1		66		
29	F	56		4.1	1		65		
30	M	57		6.3	1		126		0
31	F	58		9.0	1		76		
32	M	60		4.9	1		78		
33	M	60		5.6	1		120		77
34	M	60		9.7	1		26		
35	M	61	65	13.5	3	3	5, 4	16	0
36	F	64		2.7	1		71		31
37	M	69		11.5	2		13		0
38	M	73		5.4	1		134		1

M, male; F, female; BT, before treatment; AT, after treatment; 1 = no fibrosis; 2 = fibrosis; 3 = cirrhosis; * Percentage of $TIA\text{-}1^+$ cells within the $CD8^+$ subset; ND, not determined.

The numbers of $CD8^+$ cells in the hepatic lobuli in the all group of HH patients varied from 4 to 134 per mm^2 and did not differ significantly from those seen in normal control livers (28 to 115 $CD8^+$ cells per mm^2). However, in HH patients with cirrhosis or fibrosis the number of $CD8^+$ cells per mm^2 in the liver lobuli (median cirrhosis: 12, range: 5-36; fibrosis: 31, range 13-81) was significantly lower than in HH patients without fibrosis (median: 58, range: 24-134) (Figure 3.2, *P*

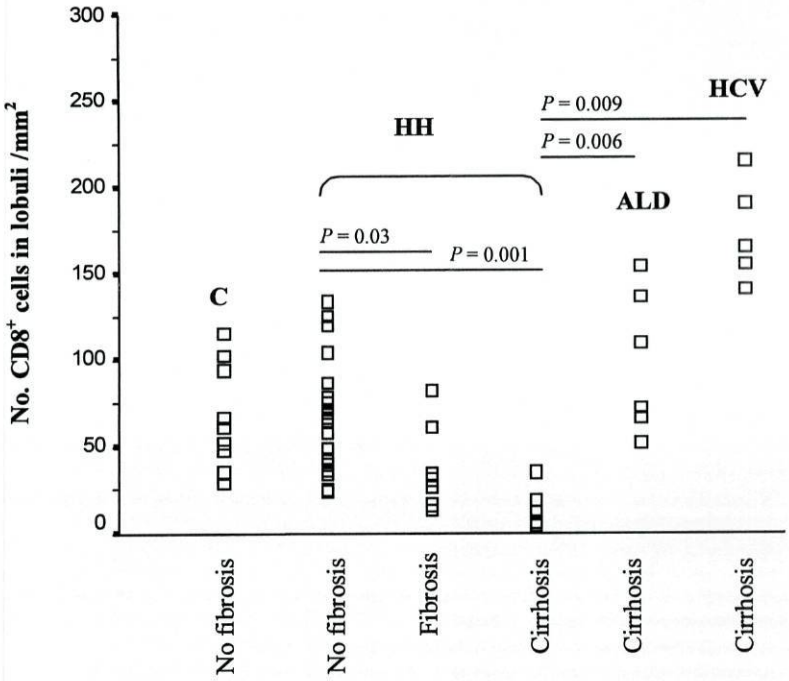


Figure 3.2 Number of $CD8^+$ cells in the lobuli per mm^2 counted in liver sections from normal controls (C), in patients with hereditary haemochromatosis (HH) and in cirrhotic controls with alcoholic liver disease (ALD) or hepatitis C (HCV). Patients with HH and cirrhosis or fibrosis had significantly lower numbers of $CD8^+$ cells in the lobuli compared to HH patients with normal liver histology (P = 0.001 and P = 0.03, respectively). Patients with cirrhosis due to ALD or HCV had significantly higher numbers of $CD8^+$ cells compared to cirrhosis due to HH (P = 0.006 and P = 0.009, respectively). P values determined by the Mann-Whitney test. See text for median and range values.

= 0.001 and P = 0.03, respectively). In contrast, the number of $CD8^+$ cells per mm^2 in patients with cirrhosis attributed to ALD (median: 91, range: 52-154) or HCV (median: 164, range: 140-215) was significantly higher than those seen in the cirrhotic livers of HH patients (P = 0.006 and P = 0.009, respectively).

Iron status and effect of iron removal on number of $CD8^+$ cells

In HH patients a negative correlation (r = - 0.54; $P < 0.001$) between iron stores and the number of $CD8^+$ cells in the lobuli was found (Figure 3.3). In six HH patients, the number of $CD8^+$ cells in the lobuli was determined before and after intensive phlebotomy treatment (Table 3.1). The number of $CD8^+$ cells in the two groups of patients, before and after treatment, was not statistically significantly different. However, in the three patients with the lowest number of $CD8^+$ cells, i.e. less than 20 cells per mm^2 (who had removed more than 10 g of iron by phlebotomies in about three years or more, and were more than 50 years old at the diagnosis) the number of $CD8^+$

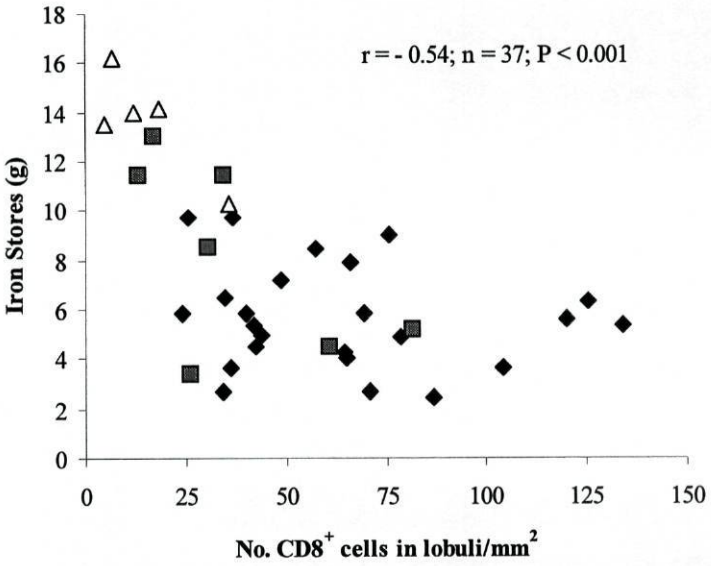


Figure 3.3 A significant negative correlation was seen between iron stores and the number of $CD8^+$ cells in the lobuli in patients with hereditary haemochromatosis. Four of the 5 patients with cirrhosis (Δ) had the lowest numbers of $CD8^+$ cells and higher iron stores (see also Figure 3.2). All HH patients were homozygous for the C282Y mutation. Triangles represent patients with cirrhosis; squares, patients with fibrosis and lozenge, patients with no fibrosis.

cells increased approximately four-fold after treatment. In the other three patients, who had removed less than 10 g of iron in about one year and were less than 50 years old at the diagnosis, the number of $CD8^+$ cells in the lobuli remained approximately the same or decreased slightly. In these six patients, two (no. 27 and 35) had cirrhosis and three (no. 10, 11 and 21) had fibrosis; no improvement of the fibrotic status was seen after intensive treatment (Table 3.1).

TIA-1$^+$ cells

To further characterize the phenotype of the $CD8^+$ cells in the liver lobuli, double immunolabeling experiments were performed in 15 HH patients and 3 controls (Table3.1), for detection of the simultaneous expression of the CD8 and TIA-1 antigens. In the majority of biopsies studied, the number of TIA-1^+ cells was higher than the number of $CD8^+$ cells (data not shown). The percentage of $CD8^+$TIA-1^+ cells in the lobuli varied considerably (0% to 77%) between patients. In three normal controls, the percentage of $CD8^+$ cells in the lobuli that was positive for TIA-1 protein was fairly constant (10, 11 and 13%). The percentage of $CD8^+$TIA-1^+ cells could not be correlated with fibrotic status, iron stores or total number of $CD8^+$ cells.

$CD8^+$ cells in the small intestine

There were significantly fewer ($P = 0.002$) $CD8^+$ cells in the lamina propria in HH patients than in controls (Table 3.2). Although the numbers of intraepithelial $CD8^+$ cells in HH patients were somehow lower than in controls, the difference was not statistically significant. No correlation was found between body iron stores and the number of $CD8^+$ cells in the small intestine ($CD8^+$ IEL or $CD8^+$ cells in the lamina propria).

Table 3.2 $CD8^+$ cell counts in the small intestine of HH patients and normal controls

Group	n	Localisation	No. $CD8^+$ cells per 100 cells§ (median, range)
HH	11	Epithelium	20 (3 - 38)
		Lamina propria	5* (3 - 7)
Controls	17	Epithelium	27 (7 - 59)
		Lamina propria	9 (4 - 18)

§No. of $CD8^+$ IEL per 100 epithelial cells (in the epithelium) or No. $CD8^+$ LP cells per 100 LP cells (in the lamina propria); *The number of $CD8^+$ cells per 100 lamina propria cells was significantly lower in HH patients than in normal controls ($P = 0.002$, Mann-Whitney test).

Analysis in the peripheral blood

In 38 HH patients studied, the level of iron stores correlated negatively with the number of peripheral blood $CD8^+$ T-lymphocytes ($r = - 0.43$; $P < 0.01$), but not with the number of $CD4^+$ T-lymphocytes ($r = 0.07$; $P > 0.05$). Furthermore, the number of $CD8^+$ cells in the peripheral blood correlated with those seen in the liver sections ($n = 37$, $r = 0.50$; $P < 0.01$). No correlation was observed between the number of $CD8^+$ cells in the peripheral blood and in the small intestine (data not shown).

Effect of age

The principal result observed, a negative correlation between iron stores and the number of hepatic $CD8^+$ cells (Figure 3.3), could be attributable to age. However, as shown in Figure 3.4, neither variations in numbers of $CD8^+$ cells (Figure 3.4a) nor individual differences in iron stores (Figure 3.4b) could be however attributed to age. Interestingly, the oldest patient in the group (no. 38, Table 3.1), aged 73 years had the highest $CD8^+$ cell counts in the liver (134 $CD8^+$ cells/mm^2), no fibrosis and moderately low iron stores (5.4 g).

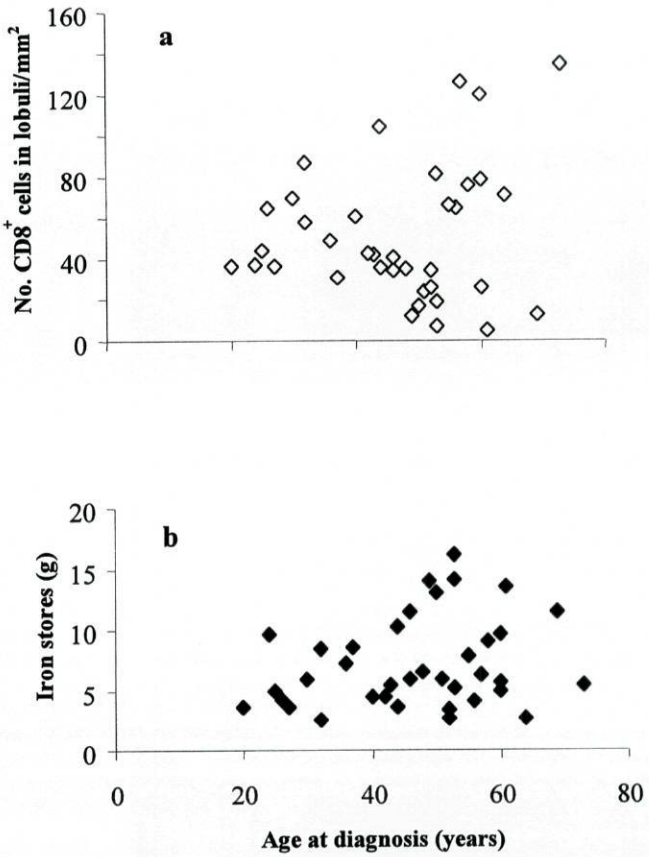


Figure 3.4 Evidence that age has no effect on numbers of $CD8^+$ cells (a, r = 0.16, NS) or on iron stores (b, r = 0.16, NS). See also Table 3.1.

DISCUSSION

The HH patients in the present study were selected according to strict criteria including homozygosity for the C282Y mutation in the *HFE* gene. Patients whose iron stores could have been altered by external factors such as alcohol, iron supplementation, transfusion or bleeding and inflammatory liver diseases were excluded. In spite of this, these HH patients had different iron stores and a very diverse clinical expression, regardless of age. Indeed, and in accordance with the results of other studies, the age of the HH patients at diagnosis could not explain the heterogeneity in the clinical expression of the disease (Porto et al., 1994; Adams et al., 1997). The amount of iron stores was negatively correlated with the number of $CD8^+$ cells in the hepatic lobuli. Moreover, in the present group of HH patients a significant negative correlation between iron

stores and the number of $CD8^+$ T-lymphocytes in the peripheral blood was found, thus confirming results obtained in other populations (Porto et al., 1994; 1997). The finding that iron stores in patients with the same mutation correlate negatively with the number of $CD8^+$ cells in the liver lobuli and in the blood, indicates that the "setting" of $CD8^+$ cell numbers may be an important indicator of progression of iron overload in HH. The fact that there were no differences in the number of $CD8^+$ cells in the liver lobuli between HH patients and normal controls confirms the conclusion reached from the studies of experimental models. In the exclusive absence of lymphocytes, like in the single *CD8* or *RAG-1* knockout mice no *spontaneous* iron overload develops (Santos et al., 1996; 1999). It appears that it is necessary to have the combination of low lymphocyte numbers and consistently high iron influx, due to genetic or other factors such as dietary or transfusional overload, to develop hepatocyte iron overload leading to organ damage. This is well illustrated in the correlation between iron stores and $CD8^+$ cell numbers seen in HH patients and by the iron overload provoked into *TCRγδ* (Ten-Elshof et al., 1999) and *RAG-1/*β_2*m* double knockout mice (Santos et al., 1999).

In HH, inflammation with an increased number of chronic inflammatory cells is not a major histological feature in the liver (Powell et al., 1994). However, it has been reported that some patients present a scarce or moderate cell infiltrate (Stal et al., 1995; Deugnier et al., 1992). In our group of patients there were no evident signs of inflammation. As stated above, we observed the lowest counts of $CD8^+$ cells in HH patients with cirrhosis. Patients with cirrhosis due to other factors had either normal or high levels of $CD8^+$ cells in the liver lobuli, confirming that the low number of $CD8^+$ cells observed in HH patients with cirrhosis could not be attributed to the cirrhosis itself. This finding is supported by other studies showing that patients with HCV infections have unchanged or increased number of $CD8^+$ cells when they have cirrhosis (Khakoo et al., 1997).

It has been shown previously that in HH patients, absolute numbers of $CD8^+$ T-lymphocytes in peripheral blood remain constant after treatment with venesection therapy (Porto et al., 1997). In the present study of liver biopsies, the number of $CD8^+$ cells in some patients remained unchanged after treatment. However, three patients, who had removed more than 10 g of iron, in about three years or more, and were more than 50 years old at diagnosis, showed an increase of about four-fold in the number of $CD8^+$ cells after treatment. This may indicate that iron loaded liver cells itself, perhaps has, to some extent, a regulatory effect on the survival of the $CD8^+$ cell subpopulation. Nevertheless, it should be stressed that these three patients had very low numbers of $CD8^+$ cells in the lobuli at diagnosis, and in spite of the increase in its $CD8^+$ cell counts after treatment when iron has been removed, their numbers remained low, specially the two with cirrhosis.

In some patients, part of the $CD8^+$ cells in the liver lobuli were also TIA-1^+. The TIA-1^+ cells that were not $CD8^+$ could be natural killer cells, neutrophils or a subset of $CD4^+$ cells (Anderson et al., 1990; Medley et al., 1996). In HH patients we found a broad range in the percentage of $CD8^+$ cells also positive for the TIA-1 molecule. The variation did not correlate, however, with iron stores or total number of $CD8^+$ cells. The possibility that a cytotoxic $CD8^+$ cell response to iron overload involving the TIA-1 molecule has thus been excluded. The possibility of $CD8^+$ cells being cytotoxic through other pathways such as Fas/FasL remains to be explored.

Finally, this study demonstrates that the low number of $CD8^+$ cells seen in the blood of some HH patients was not a consequence of "trapping" of that population in the liver, as it possibly occurs in other situations (Nuti et al., 1998). HH patients of this study were also found to have low number of $CD8^+$ cells in the lamina propria compared to normal controls, an observation compatible with the presence of a generalised lymphopenia in these patients.

It has been reported that the absolute numbers of $CD4^+$ and $CD8^+$ T-lymphocytes decrease with age (Doria and Frasca, 1997). The low numbers of $CD8^+$ cells observed in some HH patients in the liver does not seem to be a consequence of age however, since no correlation was found between the number of $CD8^+$ cells in the lobuli and age at diagnosis. A recent study in *HFE*-deficient mice revealed no abnormalities in $CD8^+$ cell numbers in lymph nodes, thymus and gut intraepithelium (Bahram et al., 1999). These results do not contradict the present study. First, Bahram and collaborators, did not characterise $CD8^+$ cells in peripheral blood, where anomalies have been reported in HH patients (Porto et al., 1994, 1997; Arosa et al., 1997) and liver (present manuscript). Second, in the study of Bahram et al., the mice have a similar genetic background, while this is not the case in the group of HH patients. It is known that the numbers of $CD8^+$ cells in humans is highly variable (Amadori et al., 1995). The first model of spontaneous iron overload was described, however, in *β2m* -/- mice. It has now been shown that crossing *β2m* -/- with *HFE* -/- the degree of iron overload is significantly increased in the double knockout mice (Levy et al., 2000). This may mimic the situation in humans, i.e., to have the mutation alone is not sufficient to develop high iron stores. Additional alterations are needed to develop more advanced iron overload.

We have shown that the clinical heterogeneity in C282Y homozygous HH patients, namely the amount of iron stores and the degree of liver damage, correlates with the number of $CD8^+$ cells in the liver lobuli, but not to the age at diagnosis. Hence, patients with increased iron absorption due to the same *HFE* mutation may develop a variable severity of expression of the disease, dependent on the number of $CD8^+$ cells in the liver lobuli. It is well known that the immune system may interfere with iron metabolism, namely through cytokines (Weiss et al., 1995). One may speculate that hepatic $CD8^+$ cells may modulate the iron efflux from the hepatocytes.

CHAPTER 4 – IRON OVERLOAD IN ATHYMIC AND EUTHYMIC RATS

ICBAS

INTRODUCTION

A role for the lymphocyte circulation in the surveillance of metals, namely iron was first postulated by de Sousa in 1978 (de Sousa et al., 1978a). The postulate predicted that abnormal accumulation of iron in tissues would act as a signal for ingress of immune cells known to contain, synthesise, and release iron-binding proteins (de Sousa et al., 1978b). Later, in a study of synovial changes after intravenous injection of ferric citrate, it was reported that an increase in transferrin saturation, led to a significant increase in the number of lymphocytes in the synovium (de Sousa et al., 1988). Conversely, iron chelation, led also to modified lymphocyte migration patterns and suppressed mononuclear cell activation, in a rat cardiac allograft model (Whitley et al., 1993). More recently, other animal models demonstrated that animals deficient in lymphocytes have defective iron homeostasis, under conditions of iron overload (Santos et al., 1999; Ten-Elshof et al., 1999). Moreover, several studies showed that in patients with hereditary haemochromatosis (HH), defective numbers of certain T lymphocyte subpopulations in peripheral blood, particularly $CD8^+$ T lymphocytes, are associated with a more severe iron overload (reviewed by de Sousa et al., 2000). Finally, we have previously shown (chapter 3) that in the liver, the degree of iron accumulation and liver damage was associated with low numbers of $CD8\alpha^+$ cells in the lobuli, while HH patients with higher number of $CD8\alpha^+$ cells had low iron stores and no fibrosis.

The CD8 molecule is present in several subpopulations of T lymphocytes, including cytotoxic T cells (Cantor et al., 1975; Ledbetter et al., 1980), $TCR\gamma\delta^+$ T cells (Lanier et al., 1987; Jitsukawa et al., 1987; Moretta et al., 1988; Goodman et al., 1988; Bonneville et al., 1988) and on NK cells (Hercend et al., 1983; Cantrell et al., 1982). In the latter subsets, $CD8^+$ cells frequently lack the β chain and mostly express CD8α homodimers. This has been shown in human CD3- natural killer cells, $TCR\gamma\delta^+$ cells and $CD4^+CD8^+$ T cells (Moebius et al., 1991), mouse thymus-independent $TCR\gamma\delta^+$ cells or $TCR\alpha\beta^+$ cells (Guy-Grand et al., 1991), mouse thymic activated $TCR\gamma\delta^+$ cells (MacDonald et al., 1990), the majority of murine peripheral $CD8^+TCR\gamma\delta^+$ T cells (Cron et al., 1989), and in IL-4-induced double positive $CD4^+CD8^+$ T cell clones (Hori et al., 1991). Furthermore, there is evidence suggesting that the expression of CD8αβ is more thymic dependent, while CD8αα expressing cells may arise extrathymically (Rocha et al., 1991, 1994; Guy-Grand et al., 1991, 1993; Murosaki et al., 1991). In rats it has been shown that most NK cells express the CD8 molecule (Cantrell et al., 1982; Lawetzky et al., 1990) and that they carry only CD8α chains (Torres-Nagel et al., 1992). In athymic rats, although some of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ subsets have the CD8αβ phenotype, a considerable fraction of these subsets express only the

CD8α chain (Torres-Nagel et al., 1992). Thus, like in mice, in rats, the expression of the CD8 heterodimer (CD8αβ) is more dependent on intrathymic maturation than that of the CD8 homodimer (CD8αα). The availability of athymic rats might thus be an opportunity to study $CD8\alpha\alpha^+$ cells, in the presence of low numbers of $CD8\alpha\beta^+$ cells.

To further investigate the possible association of $CD8^+$ cell subsets with the amount of iron stores in different tissues and the degree of hepatic iron-induced toxicity, an iron overload model has been assessed in athymic rats. Euthymic littermates were used as controls. Iron overload was provoked by a carbonyl iron-enriched diet, which has been shown to mimic the iron-loading pattern of HH, with predominant hepatocellular iron deposition in a periportal distribution (Bacon et al., 1983a; Park et al., 1987). As reviewed in chapter 1, iron in excessive amounts may lead to oxidative stress, resulting from the peroxidative damage of polyunsaturated fatty acyl moieties in membrane phospholipids (Bacon et al., 1983a, 1983b, 1985, 1986, 1990; Myers et al., 1991). In this study, the degree of *in vivo* hepatic lipid peroxidation was detected by determining the content of malondialdehyde (Pietrangelo et al., 1995).

MATERIAL AND METHODS

Animals and treatments

Male athymic nude HsdHan:rnu-rnu (Rowett nude) rats and euthymic HsdHan:rnu/+ heterozygous littermates were purchased from the Harlan Netherland (Horst, The Netherlands). The animals arrived at 5 weeks of age and were quarantined for 1 week with a chow diet (Altromin International, Lage, Germany). Some rats were sacrificed at 6 weeks of age, while others were maintained in a chow diet with or without 2.5 % (w/w) carbonyl iron (Sigma, Sweden) for 8 weeks (Figure 4.1).

All animals were kept in a room at a temperature of 21-25 °C, a relative humidity of about 50 % and on a twelve-hour light-dark cycle. Normal tap water was given to all rats. Athymic rats were kept under pathogen-free conditions, in a room with separate air ventilation and with filters. All cages, drinking bottles and the bedding material (chips of aspen, B&K Universal AB, Sollentuna, Sweden) were autoclaved.

In spite of this, the group of athymic rats which were fed with chow diet for 8 weeks (see "control" group in Figure 4.1) had an infection in the small intestine (gram positive bacteria), which was associated with lymphocytic infiltration. For this reason, this group of rats was excluded from the study. Thus, the effect of iron in the diet was analysed exclusively in euthymic

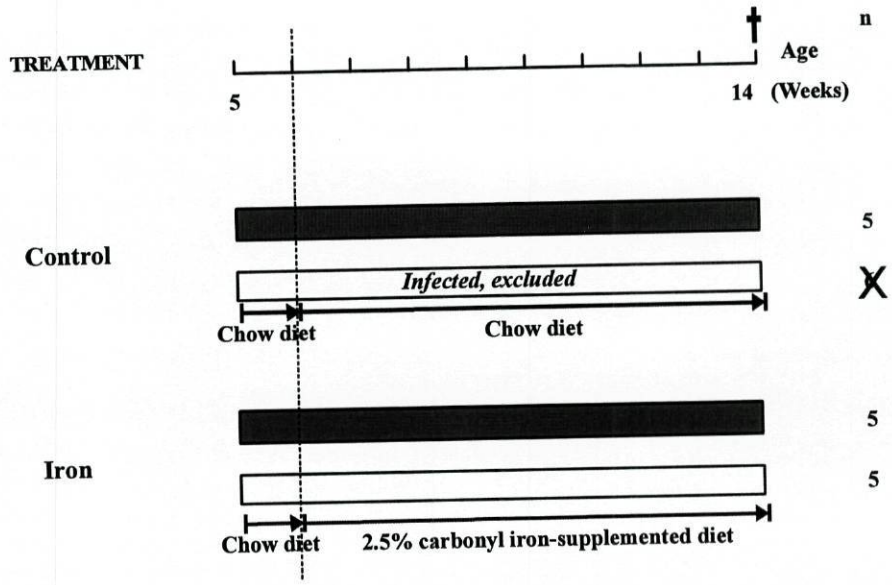


Figure 4.1 Schematic presentation of the different treatments applied. "Control" and "Iron" groups, include those rats which after the quarantine week were fed with a chow diet or 2.5% carbonyl iron-supplemented diet, respectively. n, number of rats per group; † time of sacrifice; ▬ Euthymic rats; ▭ Athymic rats.

rats. Comparison between athymic and euthymic rats was analysed only in the group of 6 weeks old rats, fed with chow diet (not shown in Figure 4.1) and in the group of rats fed with an iron-supplemented diet for 8 weeks (see "iron" groups in Figure 4.1).

The study was done with the approval of the Ethical Committee of the Karolinska Hospital.

Measurements in the serum

Serum measurement of alanine aminotransferase (ALT), iron concentration and total iron binding capacity (TIBC) were performed at the Karolinska Hospital's Department of Chemistry. Transferrin saturation was calculated from the TIBC and serum iron values.

Measurement of tissue iron content

Organ samples were weighed wet (between 50 to 100 mg wet weight), dried for 3 days at 50 °C and weighed again (dry weight). Samples were digested in 65 % HNO_3, overnight, and then diluted in a 3.5 % HNO_3 solution. Iron determination was performed in an atomic absorption spectrophotometer (Varian SpectraAA 400), using background correction. Results were expressed as μg of iron per g of dry tissue weight (μg/g dry wt).

Measurement of tissue malondialdehyde (MDA)

For the determination of hepatic malondialdehyde (MDA) content, a piece of liver was snap frozen in liquid nitrogen and stored at - 70 °C until use. Preparations of 1 % homogenates in 0.03 M Tris-HCl, pH 7.4, were performed on ice, and the resulting homogenates were centrifuged at 14000 rpm for 10 min. Supernatants were then passed through 0.2 μm filters, followed by passage through Millipore filters (Ultrafree-MC UFC 3 LGC00, Millipore Corporation, Miliford, MA, USA) using centrifugation at 14000 rpm for 10 min. The final supernatants were frozen in liquid nitrogen, and stored at –70 °C until analysis by HPLC. As a standard for MDA, 20 μM tetrahydroxypropane was used (Largilliere and Melancon, 1988) as follows: 25 μl tetrahydroxypropane were incubated with 50 ml distilled water and 100 μl 1 M HCl at 50 °C for 1 hour. Distilled water was then added to give a volume of 100 ml and the 1 mM solution obtained was passed through 0.2 μm filters. 200 μl of this solution were mixed with 9 ml 0.03 M Tris-HCl, pH 7.4 to give a 20 μM solution. From this solution, a standard curve in the interval 0.1-5 μM was prepared. The concentrations of MDA in the samples were calculated from the standard curve obtained in this interval. Acetronitrile/0.03 M Tris-HCl, pH 7.4, was used as HPLC eluant. The HPLC system consisted of a Waters 600 delivery system (1ml/min) and a Waters 717 autosampler (both Waters Chromatography Division, Millipore Corporation). Waters 486 UV detector (wavelength = 267 nm) and Waters carbohydrate analyser column (125 Å, 10 μm) were used. Data were recorded in a Waters Millenium computerised chromatography system.

Immunohistochemical staining

Small pieces of liver and spleen were dissected and transferred into a cold solution of Histocon (Histolab Products AB, Frölunda Sweden), where they were kept for some minutes. Then, each piece was snap frozen in liquid nitrogen and stored at - 70 °C. Cryostat sections, 6 μm thick, were mounted onto gelatine covered glass slides, dried for 1 hour at room temperature and frozen at - 70 °C. All specimens, were fixed in ice-cold acetone: 30 sec in 50 % acetone (diluted in distilled water) and directly after in 100 % acetone for 3 min. Slides were allowed to dry and then soaked for approximately 10 min in phosphate buffered saline, pH 7.4 (PBS). Endogenous peroxidase activity was blocked by incubating the slides in 1 % hydrogen peroxide for 15 min, in the dark. Then slides were washed three times in PBS and incubated with 2 % normal goat serum (diluted in PBS) for 20 min, to reduce background staining. In the liver, endogenous biotin was blocked by incubating sections for 15 min with avidin, and then, after washing, for 15 min with biotin (Vector Laboratories, Burlingame, CA). After washing, sections were incubated for 1 h, at

room temperature, with primary antibodies specific for selected T-cell subset markers (Table 4.1). The following rat-specific monoclonal antibodies (mAb) were used: R73 (TCRαβ), OX8 (CD8α), both kindly provided by Helena Harris from Department of Reumatology, Karolinska Hospital (KH), Sweden; V65 (TCRγδ), kindly provided by Åsa Jansson also from the Department of Reumatology of KH; OX19 (CD5) and clone 341 (CD8αβ) were commercial antibodies from Phamingen (San Diego, CA, USA) and ImmunoKontact (Frankfurt, Germany), respectively. Primary antibodies were used at concentrations 1-10 μg/ml. After 3 washings in PBS, sections were incubated for 30 min with a biotinylated goat anti-mouse (Caltag, San Francisco CA, USA) diluted 1:300 in PBS with 2 % normal goat serum (DAKO, Glostrup, Denmark). After three washes in PBS, slides were incubated for 30 min with the avidin-biotin complex (ABC, Vector Laboratories) and then, following 3 washes in PBS, the colour reaction was developed with DAB (Vector Laboratories) for 2-10 min. Slides were rinsed in tap water, counterstained with Mayer's haematoxylin (from the local pharmacy) and mounted in glycerol gelatine. An irrelevant mouse monoclonal antibody (Clone DAK-GO1, DAKO) was used as negative control. The specificity of DAK-GO1 is directed towards *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues. Sections of spleen were used as positive controls, and also to determine the optimal titers of the monoclonal antibodies used.

Iron staining in the liver

The presence of iron in the liver was detected by the Perls' blue staining method. Briefly, slides were directly transferred from - 70 °C into cold formaldehyde 2 %, and were fixed for 20 min. After rinsing 3 times in distilled water, slides were incubated for 30 min in a solution 1:1 potassium ferrocyanide 2 %: HCl 2 %. Then, sections were rinsed once in distilled water and counterstained for 5 min in "Romeis solution" (from the local pharmacy). Finally, sections were rinsed in distilled water, dehydrated in gradient alcohol's and mounted in *Entellan* (Merck, Germany).

Table 4.1 Monoclonal antibodies used in the immunohistochemical staining procedures, and cellular expression of investigated cell markers

Antibody (clone)	Surface molecule recognised	Cell population marked	References
OX 8	CD8α chain	T cytotoxic/suppressor cells, NK cells	Brideau et al., 1980 Torres-Nagel et al., 1992 Reynolds et al., 1981
341	CD8β chain	T cytotoxic/suppressor cells (not NK cells or intestinal intraepithelial cells)	Torres-Nagel et al., 1992
RT73	TCRαβ	T cells	Huning et al., 1989 Dissen et al., 1990
V65	TCRγδ	T cells	Kuhnlein et al., 1994
OX19	CD5	Only T cells and a subset of B cells (not NK cells or TCRγδ$^+$ intestinal intraepithelial cells)	Dallman et al., 1984 Vermeer et al., 1994 Lawetzky et al., 1990 Kuhnlein et al., 1994

Quantification of positive cells

The quantification of positive cells in the liver lobuli was performed with a cross-hatched grid mounted into the ×10 eyepiece of a microscope (Nikon Eclipse E800). Positive cells within the grid area were counted with a ×40 objective (corresponding to an area of 0.0625 mm^2 in the biopsy). The number of positive cells was counted in areas across the entire liver section of each specimen. Counts were expressed as the number of positive cells per mm^2.

Statistical analysis

Results are presented as the mean and standard deviation (SD). Student's t test was used to assess the significance of the differences between groups. $P < 0.05$ were considered significant.

RESULTS

Cell subsets in the liver

Athymic and euthymic rats maintained with chow diet (6 weeks aged group)

As specified in material and methods, the comparison between athymic and euthymic rats maintained in a chow diet could only be done at 6 weeks, i.e. one week after the animals arrived to the lab (Figure 4.1). As shown in Table 4.2, at 6 weeks athymic rats showed significantly higher numbers of $CD8\alpha^+$ cells per mm^2 in the liver lobuli than euthymic littermates (46.32 ± 11.31 vs. 31.73 ± 5.11, respectively, $P = 0.03$). On the contrary, athymic rats showed significantly lower numbers of $CD8\alpha\beta^+$ cells, compared to euthymic rats (athymic vs. euthymic: 0.38 ± 0.25 vs. 2.42 ± 1.27, $P < 0.05$).

Table 4.2 Comparison of CD8 positive cell subsets, stained by immunohistochemistry in the liver of 6-week-old athymic and euthymic rats

Genotype		CD8 T cell subset (number of positive cells per mm^2 in the liver lobuli)	
	n	CD8α	CD8αβ
Athymic	5	46.32 ± 11.31¶	0.38 ± 0.25§
Euthymic	5	31.73 ± 5.11	2.42 ± 1.27

Abbreviations: n, number of rats per group. Values are presented as mean ± SD. Student's t test was used for comparison between groups: ¶ $P < 0.05$, compared with euthymic rats group.

Comparison of $CD8^+$ cell subsets between athymic and euthymic rats maintained with iron-supplemented diet for 8 weeks

As shown in Table 4.3, following iron-supplementation in the diet for 8 weeks, the higher numbers of $CD8\alpha^+$ cells per mm^2 in the liver lobuli in athymic than euthymic rats continued to be observed (40.93 ± 10.50 vs. 27.12 ± 3.83, respectively, $P = 0.025$). Regarding the number of $CD8\alpha\beta^+$ cells per mm^2 in the liver lobuli, there were no significant differences between iron-loaded athymic and euthymic rats ($CD8\alpha\beta^+$: 0.95 ± 1.04 vs. 1.81 ± 0.68, respectively, Table 4.3), contrary to what been observed at 6 weeks in animals kept in the chow diet (Table 4.2).

Table 4.3 Comparison of CD8 positive cell subsets, stained by immunohistochemistry in the liver of athymic and euthymic rats fed with an iron-enriched diet or chow diet for 8 week

Genotype Treatment (diet)	n	CD8 T cell subset (number of positive cells per mm^2 in the liver lobuli) CD8α	 CD8αβ
Athymic **iron-enriched**	5	40.93 ± 10.50¶	0.95 ± 1.04
Euthymic **iron-enriched**	5	27.12 ± 3.83§	1.81 ± 0.68
chow (control)	5	16.29 ± 4.76	1.40 ± 0.16

Abbreviations: n, number of rats per group. Values are presented as mean ± SD. Student's t test was used for comparison between groups: ¶ $P < 0.05$, compared with euthymic rats group which have had the same treatment (iron-enriched diet for 8 weeks); § $P < 0.05$, compared with euthymic rats that had chow diet for the same period of time (8 weeks).

Comparison of $CD8^+$ cell subsets between euthymic rats maintained with an iron-enriched or chow diet for 8 weeks

Following iron-supplementation diet for 8 weeks, euthymic rats increased significantly the number of $CD8\alpha^+$ cells per mm^2 in the liver lobuli, when compared with the control group, which was fed with chow diet for the same period of time (27.12 ± 3.83 vs. 16.29, respectively, $P = 0.004$). No significant difference was noted between the numbers of $CD8\alpha\beta^+$ cells (iron-supplemented vs. chow diet: 1.81 ± 0.68 vs. 1.40 ± 0.16) (see Table 4.3).

Iron, liver damage and lipid peroxidation

Athymic and euthymic rats (maintained with an iron-enriched diet for 8 weeks)

Iron parameters

To characterise iron status in athymic and euthymic rats, iron contents in serum and in different tissues were analysed after feeding the rats for 8 weeks with a 2.5 % (w/w) carbonyl-iron supplemented diet. In the serum no significant differences in iron concentration or in transferrin saturation were observed between the two groups (Table 4.4). In the liver athymic rats presented slightly higher hepatic iron levels, as compared to euthymic rats (5451.1 ± 715.7 vs. 4641.8 ± 1076.1, respectively, Table 4.4). However, this difference did not reach statistical significance ($P = 0.199$). In the group of euthymic rats only one rat had 6415.58 μg/g dry wt, while the remaining had all less than 4800 μg/g dry wt. In contrast, all athymic rats, except one, presented more than 4600 μg/g dry wt in the liver. Athymic rats showed slightly lower splenic iron accumulation than

euthymic rats (5028.2 ± 880.3 vs. 7544.9 ± 1413.8, respectively, Table 4.4). However, this difference was not statistically significant ($P = 0.08$).

Table 4.4 Serum and tissue iron concentration

Genotype		Serum		Tissue iron (μg/g dry wt)					L/S
Treatment	n	Iron, μmol/L	Tf Sat, %	Liver	Spleen	Heart	Pancreas	Kidney	
Euthymic									
Iron‡	5	60.6 ± 10.6	83.4 ± 18.1	4641.8 ± 1076.1	7544.9 ± 1413.8	298.7 ± 26.5	143.6 ± 69.2	475.7 ± 47.8	0.62 ± 0.15
Athymic									
Iron‡	5	55.0 ± 16.4	66.6 ± 20.2	5451.1 ± 715.7	5028.2 ± 880.3	281.5 ± 29.7	160.0 ± 39.6	507.2 ± 26.8	0.92 ± 0.20¶

‡ rats fed with a 2.5% (w/w) carbonyl iron supplemented diet for 8 weeks after the quarantine week. Abbreviations: n, number of rats per group; Tf Sat, transferrin saturation; L/S, liver to spleen iron ratio. Values are presented as mean ± SD.
Student's t test was used for comparison between groups: ¶ $P < 0.05$, compared with euthymic rats group, which have had the same treatment.

The liver to spleen ratio (L/S) is an indicator of the predominant site of iron storage. A liver to spleen ratio greater than 1 indicates predominantly parenchymal iron loading, while a ratio of less than 1 indicates predominantly reticuloendothelial iron loading (Prophet et al., 1992). When fed with iron-supplemented diet for 8 weeks, athymic rats showed a significantly higher liver to spleen ratio than euthymic rats (0.92 ± 0.20 vs. 0.62 ± 0.15, respectively, $P = 0.031$, Table 4.4).

As shown in table 4.4, there were no significant differences in the level of iron accumulation in the heart, pancreas or kidney, between athymic and euthymic rats.

Hepatic iron distribution

Following iron-supplementation diet for 8 weeks, all athymic rats had considerable iron deposition, predominantly in periportal areas. All euthymic rats showed also liver iron staining, although four out of five rats demonstrated a lesser degree of iron staining intensity, than athymic rats. This was not quantified. In both groups, iron loaded hepatocytes showed a periportal to centrolobular decreasing gradient in iron staining. Iron loaded Kupffer cells, although present in both groups, were more clearly visible in euthymic rats (Figure 4.2).

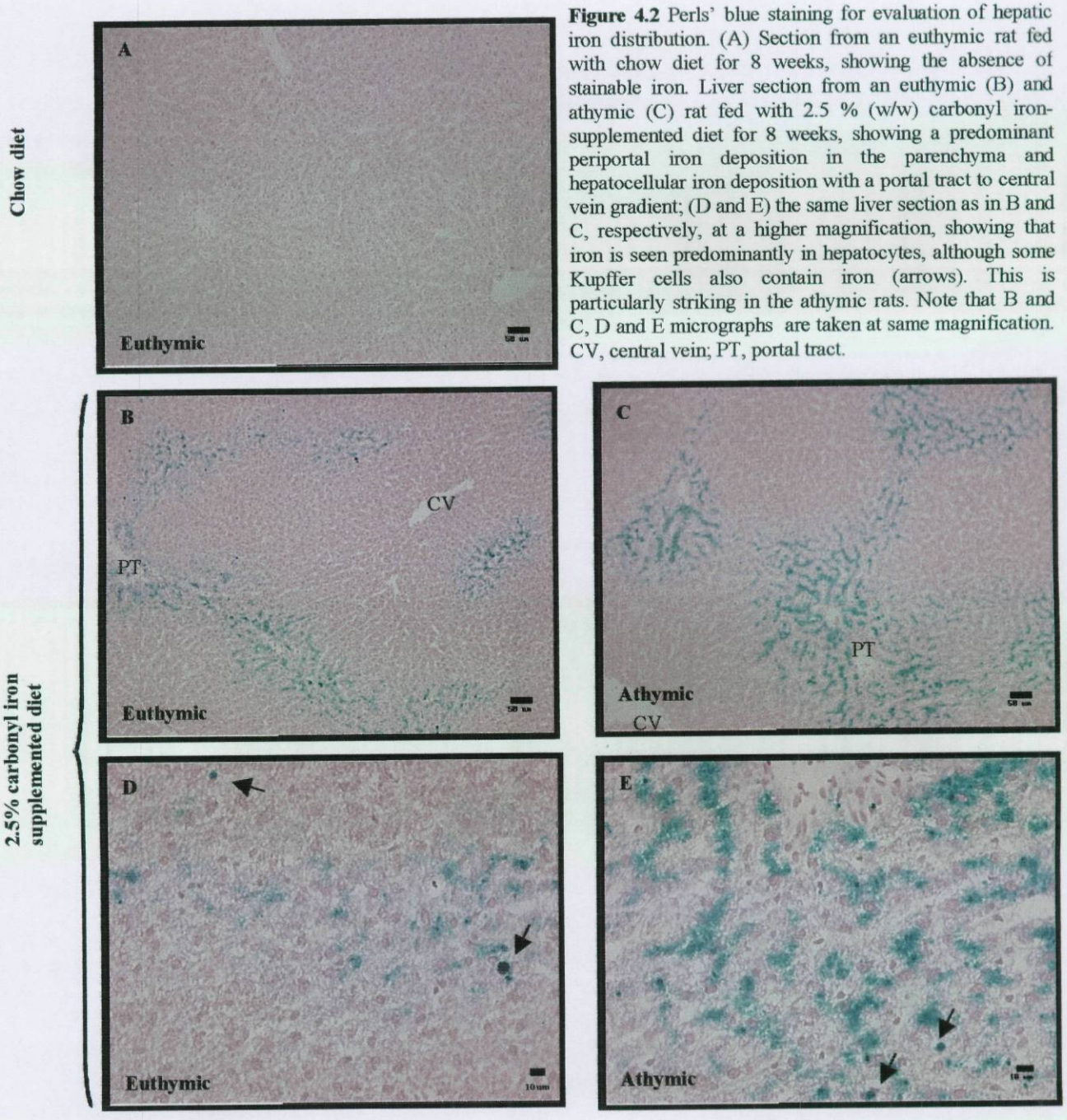


Figure 4.2 Perls' blue staining for evaluation of hepatic iron distribution. (A) Section from an euthymic rat fed with chow diet for 8 weeks, showing the absence of stainable iron. Liver section from an euthymic (B) and athymic (C) rat fed with 2.5 % (w/w) carbonyl iron-supplemented diet for 8 weeks, showing a predominant periportal iron deposition in the parenchyma and hepatocellular iron deposition with a portal tract to central vein gradient; (D and E) the same liver section as in B and C, respectively, at a higher magnification, showing that iron is seen predominantly in hepatocytes, although some Kupffer cells also contain iron (arrows). This is particularly striking in the athymic rats. Note that B and C, D and E micrographs are taken at same magnification. CV, central vein; PT, portal tract.

Liver damage and hepatic lipid peroxidation

Serum alanine aminotransferase (ALT) is present in low concentration in tissues other then liver, in which it is confined to the hepatocyte cytoplasm. High serum levels of this enzyme are therefore considered specific for hepatic damage. ALT is released from damaged cells, due to an increased permeability of cell membrane or to cell necrosis. Following iron-supplemented diet for 8 weeks, athymic rats showed higher, although not statistically significant, levels of ALT compared to euthymic littermates (0.97 ± 0.18 vs. 0.72 ± 0.17 μkatal/L, respectively, $P = 0.057$). In both groups, there was a considerable variation between individual rats.

After iron-enriched diet, MDA content in the liver was not significantly different between athymic and euthymic rats (0.17 ± 0.11 vs. 0.22 ± 0.12 μM, respectively).

Effect of iron in the diet for 8 weeks (euthymic groups)

As can be seen in euthymic rats, iron provided in the diet for 8 weeks, led to a significant increase in the majority of iron parameters tested. Thus, serum iron concentration and in the transferrin saturation increased significantly (iron-supplemented vs. chow diet: 66.6 ± 10.6 vs. 32.8 ± 4.4 μmol/L, $P = 0.001$; 83.8 ± 18.1 vs. 35.3 ± 2.1 %, and $P = 0.004$, respectively). Iron feeding, led also to a significant increase, of fourteen-fold in hepatic and four-fold in splenic iron content (iron-supplemented vs. chow diet: 4641.8 ± 1076.1 vs. 322.2 ± 23.5 μg/g dry wt, $P = 0.001$ and 7544.9 ± 1413.8 vs. 1875.5 ± 304.9 μg/g dry wt, $P < 0.001$, respectively). In the heart and in the kidney there was also a significant increase in the iron concentration (iron-supplemented vs. chow diet: 298.7 ± 26.5 vs. 247.2 ± 17.3 μg/g dry wt, $P = 0.007$ and 475.7 ± 47.8 vs. 249.9 ± 22.5 μg/g dry wt, $P < 0.001$, respectively). In the pancreas, there was an increase in iron content, although not statistically significant (iron-supplemented vs. chow diet: 143.6 ± 69.2 vs. 74.3 ± 23.4 μg/g dry wt, $P = 0.067$). Iron feeding in the diet, resulted in a significant increase in the liver to spleen iron ratio (iron-supplemented vs. chow diet: 0.62 ± 0.15 vs. 0.17 ± 0.02, $P < 0.001$).

Concerning hepatic iron distribution in the liver, no detectable iron was seen in any of the rats fed with chow diet (Figure 4.2A), while following iron-enriched diet, iron could be detected (Figure 4.2B-E). See above for a more detailed description of iron distribution in the liver.

However, the presence of iron in the diet did not lead to a significant change in the levels of ALT in euthymic rats (iron-supplemented vs. chow diet: 0.72 ± 0.17 vs. 0.66 ± 0.03 μkatal/L).

Euthymic rats fed with iron-supplemented diet demonstrated an increase in hepatic MDA concentration, however not statistically significant (iron-supplemented vs. chow diet: 0.22 ± 0.12 vs. 0.10 ± 0.05 μM, $P = 0.095$).

DISCUSSION

As expected, an increase in body iron stores in the serum and in tissues was achieved by dietary introduction of carbonyl iron (Nielsen et al., 1993). After two months of dietary iron-supplementation, a gradient in parenchymal cell iron from periportal hepatocytes decreasing towards the hepatic venule was observed, resembling the pattern associated with HH. This confirms previous studies (Park et al., 1987). However, in our model, iron deposition in the spleen was greater than in the liver, after two months of iron-enriched diet, while in other studies iron was primarily accumulated in the liver, and only after longer periods (more than 5 months) of treatment, iron deposition in the spleen became greater than in the liver (Park et al., 1987). This may be caused by the different genetic background of the rats used in this study. In fact, there is evidence indicating that the genetic background of the animal used is of great importance in the expression of iron loading (Pietrangelo et al., 1990; Leboeuf et al., 1995; Ten-Elshof et al., 1999).

Evidence of *in vivo* hepatic lipid peroxidation in experimental chronic iron overload models has been previously shown (Bacon et al., 1983a). In our study, only a tendency for increase in the hepatic MDA concentration (which is indicative of lipid peroxidation) was observed in livers of rats treated with iron-enriched diet. An explanation could be that in our model only a modest increase in liver iron accumulation was observed, while in the model from Bacon and colleagues, a higher hepatic iron content was observed. In fact, it was shown that in the liver (Bacon et al., 1983b), and that in particular organelles such as in mitochondria (Bacon et al., 1985) and in microsomes (Bacon et al., 1986), critical "thresholds" of iron levels must be exceeded in order to induce lipid peroxidation.

In view that no substantial differences in the hepatic susceptibility to iron overload between athymic and euthymic rats treated with iron-enriched diet were observed, no clear conclusions can be infer whether $CD8\alpha^+$ or $CD8\alpha\beta^+$ cell subset may have a preferential role in regulating hepatic iron accumulation or liver damage. However, interestingly, the numbers of $CD8\alpha\alpha^+$ cells in the liver (characterising NK or NK-T cells) were significantly higher in athymic rats than in euthymic littermates (whereas other cell subsets such as $CD8\alpha\beta^+$, $TCR\alpha\beta^+$ or $CD5^+$ cells (not shown), which are more thymic dependent were in lower numbers in athymic rats at 6 week age). Extrathymic proliferation within the liver might be an important *in vivo* source of these cells, which is in agreement with other studies (Abo et al., 1995; Suzuki et al., 1998). Moreover, in euthymic rats there was an increase in the hepatic number $CD8\alpha\alpha^+$ cells after iron-enriched diet, indicating that iron could be one controlling factor of the hepatic numbers of these cells. Several mechanisms may be proposed to account for this *in situ* accumulation: proliferation of resident cells and/or recruitment or "trapping" from the bone marrow-derived cells (Suzuki et al., 1998;

Ishihara et al., 1999). Thus, the observations referred above, suggest that liver and iron may be important components for the numbers of CD8αα$^+$ cells. Macrophages have able to secret TNF-α after erythrophagocytosis (Costa et al., 1998). On contrary, iron-chelation was found to inhibit profoundly TNF-α production in rats treated with LPS, suggesting that iron chelation inhibits macrophage activation (Whitley et al., 1993). Interestingly, this cytokine has been shown to have co-mitogenic properties onto T cells (Costa et al., 1998) and to be the principal cytokine involved in the recruitment of NK cells to the liver parenchyma (Pilaro et al., 1994). In addition, a novel "antigen-independent" mechanism of naive T cell activation by cytokines (TNF-α, IL-2 and IL-6) was also described (Unutmaz et al., 1995). Altogether, the observations from the present study open new possible interactions between iron metabolism, macrophages and lymphocytes within the liver environment, which remain to be further explored.

Finally, athymic rats fed with an iron-supplemented diet demonstrated a significant higher iron accumulation in the liver, and lower capacity to this metal in the spleen (comparing to the euthymic littermates). Thus, immune system composition (the presence vs. absence of thymic-dependent lymphocytes) seems to have a contribution to the iron loading pattern and accumulation in the body.

CHAPTER 5 – GENERAL DISCUSSION AND CONCLUSIONS

HETEROGENEITY OF IRON ACCUMULATION IN HH IS NOT EXPLAINED BY AGE OR *HFE*

HH is now recognised as being the most common autosomic recessive disorder in Caucasians (Merryweather-Clarke et al., 1997), characterised by increased iron absorption through the small intestine, leading to excessive accumulation of this metal in several tissues, including the liver (Powell et al., 1994). The importance of understanding how the immune system may control iron accumulation or toxicity caused by this metal was reinforced after the discovery of the gene of HH (*HFE*) in 1996 (Feder et al., 1996), since the mutations in this gene fail to explain the heterogeneity in the expression of this disease. Other gene(s) and their products are therefore expected to be implicated in HH. In this study, we confirm that HH patients, despite having the same mutations in this gene (most of the HH Swedish patients are homozygotes for the C282Y mutation in the *HFE* gene, chapter 2) accumulate different amounts of iron in the body, which could not be explained by the age at diagnosis of the patient (chapter 2 and 3). Moreover, in our group of HH patients there was one, who did not reveal any of the two main mutations in the *HFE* gene, but had fulfilled the criteria of diagnosis of HH (chapter 2), indicating that other gene(s) may be involved in the development if this disease. Although we have not investigated further whether this patient had other mutations in the *HFE* gene, another group have recently reported that some HH patients have no mutations in this gene (Pietrangelo et al., 1999). Therefore, this substantiates that mutations in other gene(s) may lead to iron overload indistinguishable of HH. Altogether, this indicates that there are other factors, which may regulate iron metabolism in HH.

HETEROGENEITY IN THE CLINICAL EXPRESSION OF HH IS ASSOCIATED WITH THE NUMBERS OF $CD8^+$ CELLS

Serial studies in Portuguese HH patients, have shown that lymphocytes, particularly the $CD8^+$ cell subset, are likely to modify the severity of iron overload (reviewed by de Sousa et al., 2000). These studies were performed in lymphocytes from peripheral blood of HH patients. These studies however, did not acertain whether lymphocyte numbers in other tissues correlated with clinical severity. The results of this thesis support and extend the earlier observations (chapter 3) in another HH population, by showing that defective $CD8^+$ cell numbers in the peripheral blood were associated with higher iron stores (chapter 3). Importantly, this was observed in a group of HH patients, who had the same genotype (C282Y homozygotes) and in whom several other factors that might influence the degree of iron accumulation had been excluded. Furthermore, the

observation that low number of $CD8^+$ cells in the liver of this very "strict" group of patients, was related to higher iron stores and liver damage (cirrhosis), indicates that this cell subset may have a role in the regulation of hepatic iron accumulation and toxicity, under deregulated iron absorption such as in HH (chapter 3). Thus, the analysis of $CD8^+$ cells in the liver may become an additional predictive marker for the clinical course of HH.

In order to examine a possible cytotoxic function for the $CD8^+$ cells observed in the liver of HH, a double immunohistochemical method for the simultaneous detection of CD8 and TIA-1 markers was developed. The TIA-1 molecule has been shown to induce apoptosis in target cells, which is a programmed form of cell death that serves to eliminate damaged cells from the body, without promoting inflammation. We have found a broad range in the percentage of $CD8^+$ cells also positive for TIA-1 molecule, which did not correlate however, with iron stores or total number of $CD8^+$ cells. Thus, the possibility that a cytotoxic $CD8^+$ cell response involving the TIA-1 molecule was excluded (chapter 3). The possibility that other mechanisms of $CD8^+$ cells cytotoxicity, such as Fas/FasL, or NK activity have a role in HH remains to be explored. Low cytotoxicity *in vitro* activity in $CD8^+$ CTL from peripheral blood of HH patients has been reported previously (Arosa et al., 1997).

LOW NUMBERS OF $CD8^+$ CELLS IN HH ARE NOT RELATED TO SEVERAL FACTORS

Low numbers of $CD8^+$ cells in the blood could be attributable to preferential migration to certain organs such as liver, as occurs in some subsets in other diseases such as in chronic HCV infection (Nuti et al., 1998). However, we have shown that this was not the case in HH, i.e., low numbers of $CD8^+$ cells in the blood were associated with low numbers of this cell subset in the liver (chapter 3). These patients revealed also low numbers of $CD8^+$ cells in the lamina propria of small intestine when compared to normal controls, suggesting that in some of those patients, there was a sort of generalised low number of $CD8^+$ lymphocytes, which concurs with results from other studies (reviewed by de Sousa et al., 2000).

In six HH patients, whose $CD8^+$ cells in the liver could be quantified before and after iron depletion in the body by intensive phlebotomy treatment, revealed no major changes in the number of this cell subset. Although, three patients who were more than 50 years old, and had removed more than 10 g of iron in more than three years, demonstrated an increase of about four-fold in the number of $CD8^+$ cells in the liver after treatment, one should stress that their lymphocyte numbers remained low after iron removal (chapter 3). That lymphocyte numbers in HH exhibit a markedly constancy after removing iron, is in accordance with earlier studies (Reimao et al., 1991; Porto et al., 1994, 1997).

The fact that patients with cirrhosis caused by ALD or HCV had higher numbers of $CD8^+$ cells in the liver than patients with cirrhosis due to HH, indicates that the low number of $CD8^+$ cells observed in the latter could not be attributed to the cirrhosis *per se* (chapter 3).

Finally, although it has been reported that in humans the absolute numbers of $CD4^+$ and $CD8^+$ T-lymphocytes in peripheral blood decrease with age (Doria and Frasca, 1997), in the present study the low numbers of $CD8^+$ cells observed in some of the HH could not be correlated to age either, since no correlation was found between the number of $CD8^+$ cells in the liver and the age at diagnosis (chapter 3).

IRON OVERLOAD IN ATHYMIC AND EUTHYMIC RATS AND FINAL CONSIDERATIONS

Athymic rats fed with an iron-supplemented diet demonstrated a significant higher iron accumulation in the liver, and lower capacity to this metal in the spleen (comparing to the euthymic littermates), suggesting that thymus-derived cells influence the pattern of tissue iron deposition (chapter 4).

One interesting result from this study was that the number of $CD8\alpha\alpha^+$ cells in the liver (characterising NK or NK-T cells) was significantly higher in athymic rats than in euthymic littermates (chapter 4). Extrathymic proliferation within the liver might be an important *in vivo* source of these cells, which is in agreement with other studies (Abo et al., 1995; Suzuki et al., 1998). In addition, an increase in the hepatic number of $CD8\alpha\alpha^+$ cells following iron-supplemented diet was observed in euthymic rats, indicating that iron could be one controlling factor of the hepatic number of these cells. Altogether, these observations suggest that liver and iron may be important factors for the "setting" of $CD8\alpha\alpha^+$ cells. The *in situ* accumulation of these cells in the liver might be a result of proliferation of resident cells and/or recruitment or "trapping" from the bone marrow-derived cells (Suzuki et al., 1998; Ishihara et al., 1999). Together with the spleen, liver is a major tissue for recycling of senescent erythocytes (task carried out by Kupffer cells). Moreover, it was reported that macrophages are able to secret TNF-α after erythrophagocytosis (Costa et al., 1998). On contrary, iron-chelation was found to inhibit profoundly TNF-α production in rats treated with LPS, suggesting that iron chelation inhibits macrophage activation (Whitley et al., 1993). Interestingly, this cytokine has been shown to have co-mitogenic properties onto T cells (Costa et al., 1998) and to be the principal cytokine involved in the recruitment of NK cells to the liver parenchyma (Pilaro et al., 1994). On the other hand, in it is known that monocytes from HH patients have an impared release of TNF-α (Gordeuk et al., 1992). This could be, in part, one additional cause for the defective numbers observed in HH

patients. Hence, novel and challenging interactions between iron metabolism, lymphocytes and macophages within the liver environment remained to be explored in the future.

One relevant issue is to characterise further which lymphocyte subsets may regulate iron-induced toxicity caused by excessive iron deposition in hepatocytes, as well as how would lymphocytes carry out those protection functions. The use of controlled *in vitro* experiments could help to overcome, at least in part, some of the problems related to *in vivo* studies. For instances to determine more precisely the lymphocyte populations involved in protective immunity against iron-induced toxicity, *in vitro* activation of lymphocytes by iron loaded, hepatocyte cell lines or cultured hepatocytes, could be performed (Carayon and Bord, 1992; Paroli et al., 1994; Tong et al., 1994). This type of studies could give also insights into possible mechanisms of lymphocyte action, by analysing their production of cytokine or other mediators which may confer a protective function of lymphocytes in conditions of iron burden. In addition, this type of *in vitro* co-culture could help to understand how lymphocytes could regulate iron metabolism in the hepatocytes, or iron-caused toxicity (measured by the leakage of lactate dehydrogenase or potassium, for instance). Nevertheless, although the use of isolated cells can answer specific questions about putative mechanisms, the main disadvantage of *in vitro* experiments versus *in vivo* experiments are the extrapolation problems of the results from the first ones to the conditions of the intact organism. Thus, I would like to finish pointing out that, *in vivo* studies, such as those in the present investigation, albeit being more difficult to interpret, provide invaluable "real" information. Ideally, analyses of cell subsets and molecules involved in putative mechanisms would be tested in controlled *in vitro* experiments, and then the most evident "players" would be analysed in *in vivo* studies.

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APPENDIX I – DETECTION OF CYTOKINE PRODUCING CELLS IN HUMAN LIVER: TECHNICAL CONSIDERATIONS

PREAMBLE

As discussed in the general introduction, cytokines might be responsible for the mechanism involved in the interaction between lymphocytes and iron stores. It was of the original aims to look into this mechanism. The present appendix represents the technical efforts undertook to visualise cytokine-producing cells in the liver. It was not successful. A considerable amount of work was, however, put into it and seemed that might be valuable to describe it.

INTRODUCTION

In order to detect ongoing *in vivo* cell-producing cytokines, a method based on immunohistochemical intracellular staining of cytokines by means of a combined technique of fixation and permeabilisation (Sander et al., 1991), has been applied to the liver tissue. This method has previously been used in human tonsils (Andersson et al., 1994), rectal biopsies (Raqib et al., 1995), synovial biopsy specimens (Ulfgren et al., 1995), muscle tissue (Lundberg et al., 1997) and atherosclerotic plaque (Frostegard et al., 1999). In this protocol, the handling and staining of the detergent exposed sections preserves the morphology of the intracellular accumulated cytokines in a unique way. Fixation with aldehyde provides a significant advantage over acetone, in terms of morphological maintenance of the intracellular cytokine producing organelles. Saponins (plant glycosides with high affinity for cholesterol) have been used to permeabilise cells by intercalating cell membranes in a reversible fashion to replace cholesterol. As saponin interacts predominantly with cholesterol alone, much of the morphology of the membrane structure of the cells fixed with formaldehyde is found to remain intact when examined under the microscope (Sander et al., 1991). To analyse the presence of cytokine producing cells at the protein level would be more informative than to detect mRNA expressing cells, since detected mRNA levels do not necessarily correlate with levels of secreted protein (Raqib et al., 1996). However, because certain drawbacks appeared when cytokines were analysed at the protein level by immunohistochemistry in the liver, cytokine mRNA expressing cells (TNF-α and IFN-γ) were evaluated with the *in situ* hybridisation technique.

MATERIAL AND METHODS

Immunohistochemical detection of intracellular cytokines on formaldehyde-fixed frozen sections

Tissues, fixation and preparation

Glass slides were washed in ethanol 95 %, and let dry. Then slides were dipped into a solution 1:4 gelatine 2 % (Merck, Darmstadt, Germany): chromium (III) potassium sulphate dodecahydrate 0.067 % (Merck), at 56 °C, dried overnight, and finally stored at 4 °C until use. As a positive control a fresh surgical palatine tonsil specimen obtained from a child with recurrent tonsillitis was used. This biopsy as well as liver biopsies from a patient with chronic HCV infection were placed in cold Histocon for some minutes (Histolab Products, AB, V. Frölunda, Sweden), snap frozen in liquid nitrogen and stored at - 70 °C. Tissues were embedded in OCT-compound (Tissue-TEK, Baxter, UK) and cryostat sections were cut at 6 μm thickness, mounted on chromium potassium sulphate and gelatine-coated glass slides, and dried at room temperature for ½ to 1 h. Then sections were fixed for 20 min in cold formaldehyde 2 % (Sigma Chemicals, St Louis, MO) diluted in cold phosphate-buffered saline (PBS), washed 3 times for 5 min in PBS, dried for 30 min and finally stored at - 70 °C.

Antibodies

Monoclonal antibodies used: a mixture of mouse monoclonal antibodies (mAb) anti-TNF-α MAb1 (2.5 μg/ml) and MAb11 (3.5 μg/ml) (PharMingen, San Diego, CA) or of anti-IFN-γ DIK-1 (2.5 μg/ml) and 7-B-6 (2.5 μg/ml) (Mabtech AB, Nacka, Sweden) were used. As negative control a irrelevant mouse mAb (clone DAK-GO1, DAKO, Glostrup, Denmark) was used. The antibody specificity of DAK-GO1 is directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues.

APAAP method

Frozen sections were placed in cold tris-buffered saline (TBS), supplemented with 0.1% saponin (TBS-saponin) (Sigma) for 5 min. To reduce non-specific binding, normal rabbit serum (NRS) diluted 1:10 in TBS-saponin was applied for 10 min. Then, after blotting the excess of serum, sections were incubated, overnight, with the respective individual monoclonal antibody, at room temperature. After washing in TBS-saponin, the second layer, rabbit anti-mouse

immunoglobulin code Z259 (DAKO) was applied for 30 min and then, after washing again, the mouse APAAP conjugate, code D651 (DAKO), was applied for 30 min. Incubation steps with the link antibody (rabbit anti-mouse) and the APAAP conjugate were repeated for 10 min each, to increase the intensity of the reaction. After additional washing, colour was developed with Fast-Red substrate (DAKO). Sections were counterstained in Mayer's haematoxylin and mounted in KAISER's glycerol gelatine.

ABC-HRP method

Cryostat sections were stained for intracellular production of cytokines according to the method described by Sander et al. in 1991 (Table 1). Endogenous peroxidase activity was blocked in a solution 1 % H_2O_2, dissolved in Earls buffered salt solution (EBSS, GIBCO, Ltd., Paisley, UK), supplemented with 0.1 % saponin (EBSS-saponin) (Sigma) for 15 min, in the dark. Sections were then washed three times in EBSS-saponin and thereafter blocked with avidin-saponin for 15 min and biotin-saponin for an additional 15 min (Commercial Blocking Kit, Vector Laboratories, Burlingame, CA). After three additional washes in EBSS-saponin sections were incubated overnight, at room temperature, in a humidified chamber with 50 µl cytokine-specific antibody solution diluted in EBSS-saponin. The slides were then washed thoroughly in EBSS-saponin and sections were incubated with normal goat serum (Vector Laboratories) 2 % for 15 min. A biotinylated secondary antibody was added: either biotinylated goat anti-mouse IgG adsorbed against human Ig (Caltag Lab, South San Francisco, CA) diluted 1:300 (the best dilution was determined in tonsil sections), biotin-SP-conjugated affinity purified $F(ab')_2$ fragments goat anti-mouse IgG, Fc (gamma) fragments adsorbed against human serum (Jackson ImmunoResearch Laboratories, Baltimore, PA), or biotin-SP-conjugated affinity purified $F(ab')_2$ fragments donkey anti-mouse IgG, Fc (gamma) fragments adsorbed against human serum (Jackson ImmunoResearch Laboratories), diluted in EBSS-saponin and 2 % normal goat serum, for 30 min at room temperature. Sections were washed again three times for 5 min in EBSS-saponin and 50 µl of a solution of Vectastain avidin-biotin-horseradish peroxidase (Vectastain, ABC-HP-Kit, Vector Laboratories) prepared according manufacturer's directions in EBSS-saponin were applied for 30 min at room temperature. After a final wash in EBSS-saponin, the substrate

Table 1 Protocol for immunoenzyme staining using the ABC-HRP method

Step	Reagents	Time
1.	1 % H_2O_2 in EBSS-saponin	15 min
2.	Avidin-saponin	15 min
	Biotin-saponin	15 min
3.	Primary antibody in EBSS-saponin	overnight
4.	Normal goat serum 2 % in EBSS-saponin	15 min
5.	Secondary antibody in EBSS-saponin	30 min
6.	ABC-HRP complex in EBSS-saponin	30 min
7.	Detection of HRP activity (DAB)	2-3 min

diaminobenzidine (DAB, Peroxidase Substrate Kit, Vector Laboratories) was added. The reaction was stopped after 2-3 min by three washes in EBSS-saponin, after which sections were counterstained briefly in Mayer's haematoxylin and mounted in KAISER's glycerol gelatine.

In situ hybridisation

The method performed was adapted from previously described protocols (Dagerlind et al., 1992; Link et al., 1994).

Tissues and PBMC preparation

A total of 25 samples were tested as follows: liver biopsies - 5 from HH patients without fibrosis, 3 from HH patients with fibrosis or cirrhosis, 5 from alcoholics with no fibrosis and 5 from alcoholics with fibrosis or cirrhosis, 5 controls (from 2 subjects who had undergone liver biopsy because of slightly increased alanine transaminase (ALT) values, and 3 subjects treated with methotrexate); 1 palatine tonsil specimen obtained from a child with recurrent tonsillitis; as positive control stimulated PBMC with SEB were used.

Samples referred above were prepared as follows: tonsils and liver frozen biopsies were embedded in OCT (Tissue-Tek), cut in 14 μm sections, were mounted on Superfrost Plus glass slides (Menzel Glaser, Germany) and stored at - 70 °C until use. A peripheral blood sample from a healthy blood donor was taken into a heparinised glass tube. PBMC were immediately separated by density gradient centrifugation on lymphoprep (Nycomed, Oslo, Norway). Cells from the interphase were collected and washed three times with RPMI 1640 medium (Gibco/Life Technologies, Stockholm, Sweden). The proportion of viable cells was assessed with trypan blue (cell viability achieved was > 95 %). The cells (1×10^6 PBMC/ml) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin and 60 μg/ml streptomycin, 8 μg/ml gentamicin, 1mM sodium pyruvate (Sigma), and 5 % (v/v) human AB+ serum (blood Centre, Karolinska Hospital, Stockholm, Sweden) in humidified atmosphere air containing 5 % CO_2, at 37 °C. For cytokine induction the cells were stimulated with 0.5 μg/ml of the superantigen staphylococcal enterotoxin (SEB, Sigma St. Louis, MO), for 24 h. After stimulation the cells were washed twice in sterile PBS, and 5×10^4 cells were applied onto a Superfrost Plus glass slide (Menzel Glaser), dried at 55 °C for 30 min and stored at - 20 °C.

Probes preparation

Synthetic oligonucleotide probes were labelled with ^{35}S-deoxyadenosine-5′-α-(thio)-triphosphate, using terminal deoxynucleotidyl transferase. For each cytokine a mixture of four different probes, each approximately 48 bases long, was used to increase the sensitivity of the *in situ* hybridisation. Oligonucleotide sequences were obtained from GenBank. The human TNF-α oligonucleotide probes were complementary to bases 913-960 (exon 1), 2233-2289 (exon 2), 2383-2430 (exon 3) and 2479-2526 (exon 4) (Pennica et al., 1984; GenBank accession numbers X02159, X01394), and the human IFN-γ oligonucleotide probes were complementary to bases 507-556 (exon 1), 4682-4729 (exon 2), 4660-4707 (exon 3) and 4641-4688 (exon 4) (Gray and Goeddel, 1982; GenBank accession number J00129). A mix of 48-mer sense probes (complementary to the antisense probes for each cytokine) was used (at the same concentration as the antisense probes) as a negative control to verify the specificity of the hybridisation signal. All the probes were kindly provided by Dr. Tomas Olsson from the department of neuroimmunology, Stockholm, Sweden. Oligonucleotide probes were boiled for 1 min and cooled immediately on ice. Thereafter, 0.8 μM of probe were mixed with 2.5 μl 10× cobalt reaction buffer (1.4 M potassium cacodylate pH 7.2, 300 mM Tris base 10 mM $CoCl_2$, 2 mM 2-mercaptoethanol), 7.5 μl deionized and autoclaved DEPC treated water, 12 μl ^{35}S-dATP (New England Nuclear, USA) and 1 μl Terminal Deoxynucleotidyl Transferase (19.2 U/μl, Parma-Biotech, Stockholm, Sweden). The labelling mixture was incubated at 37 °C for 2 h and subsequently purified using a Nensorb 20 column (NEN, Life Science Products, Boston MA). The radiolabelled probes, with an activity of at least 0.5×10^6 cpm/μl, were stored in 20 % ethanol containing 2.5 mM dithiotreitol (DTT) at 4 °C until use. DTT was used to avoid possible formation of disulphides bonds.

In situ hybridisation

Sections and PBMC were thawed at room temperature prior to use and then were hybridised for 18 h, at 42 °C in a humidified chamber with 10^6 cpm/100 μl of a labelled probe in a solution of 50 % formamide (Merck), 1× Denhardt´s solution (0.02 % polyvinylpyrrolidone (Sigma), 0.02 % bovine serum albumin (USB, Cleveland, USA) and 0.02 % Ficoll (Sigma), 4× SSC (0.6 M NaCl and 0.06 M sodium citrate), 1 % sarcosyl (Sigma), 0.02 M sodium phosphate buffer (pH 7.0), 10 % dextran sulphate (Pharmacia Biotech), 500 μg/ml shared and heat-denatured salmon sperm and 100 mM DTT (LKB, Bromma, Sweden).

Washing

Sections were washed in 1× SSC buffer, for 4× 15 min at 55 °C, cooled to room temperature and rinsed in distilled water. After dehydration in an ethanol gradient (60 %, 70 % and 95 %) slides were air-dried and dipped in autoradiography emulsion, NTB2 (Kodak, Rochester, NY) diluted 1:1 in distilled water and exposed in a dark box at 4 °C for 2 weeks. The slides were developed in D19 (Kodak) and fixed in UNIFIX (Kodak), each for 4 min. After fixation, slides were rinsed in tap water, counterstained in Mayer's haematoxylin, dehydrated in alcohol in concentration of 70 %, 95 %, 99.9 % and xylene. Finally, the slides were mounted in *Entellan* (Merck).

Quantification of positive cells

Slides were evaluated by light microscopy. Cells were considered positive when more than 10 or 15 developed silver grains were observed onto the cell, being the cut-off value 10 or 15 depending on the background level. Duplicate slides with 5×10^4 PBMC were counted for each cytokine. The number of cytokine mRNA expressing cells was calculated by subtracting to the number of positive cells obtained with the antisense probes, the number of positive cells obtained with the sense probes. When fewer positive cells were counted with the antisense probes, than those were with the sense probes, the number of positive cells was considered zero.

RESULTS

Immunohistochemical detection of intracellular cytokines on formaldehyde-fixed frozen sections

APAAP method is less sensitive than the ABC-HRP method

Prior to using the ABC-HRP method recommended by others (Sander et al., 1991), the APAAP method was tested, since we would like to stain liver tissue, which is rich in endogenous peroxidase and biotin. In addition, with the APAAP method, the alkaline phosphatase substrate Fast Red could be used, which by giving a red colour would be preferable to use in tissues rich in iron deposits (such as in HH patients) that have a brown colour when observed under the light microscope. Thus, serial sections from human tonsil tissue were stained using both the APAAP and the ABC-HRP methods. The latter was performed using biotinylated goat anti-mouse as secondary antibody (Table 1, step 5). In serial tonsil sections in average, less then 15 weak TNF-α or IFN-γ positive cells per section were observed when the APAAP method was used, while with the ABC-HRP method, more than 25 and strong TNF-α or IFN-γ positive cells per section could be observed. With both methods, in the negative control slide (incubated with irrelevant mouse IgG or omission of the primary antibody) none or occasional 1-2 positive cells per section were detected. Note that here, the quantification was not performed with

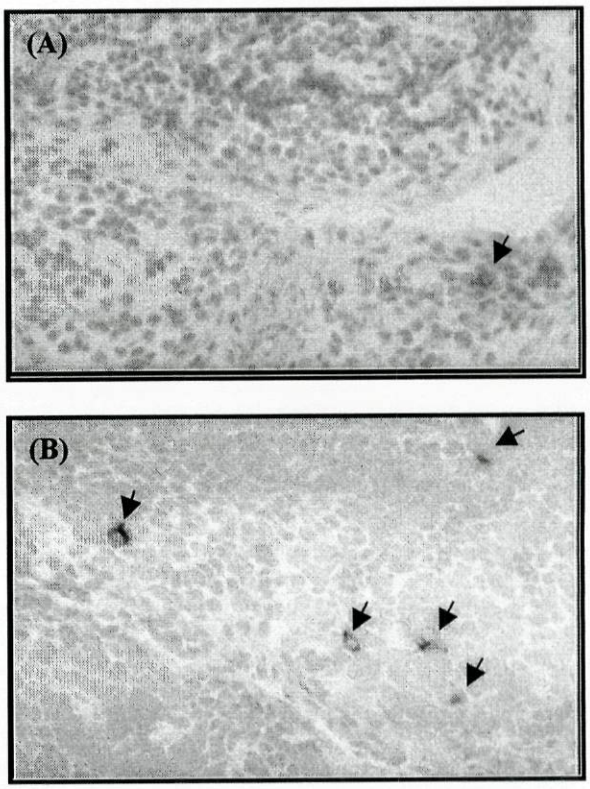


Figure 1 Comparison of sensitivity of APAAP and ABC-HRP methods. Representative sections of a frozen human tonsil from a child with recurrent tonsillitis, stained for demonstration of TNF-α producing cells using the APAAP (A) or ABC-HRP (B) methods. The ABC-HRP method revealed more and stronger stained positive cells (arrows) than the APAAP method. Counterstained in Mayer's haematoxylin. Original magnification ×250.

the detail described in the chapter 3. Since the APAAP method revealed to be less sensitive than the ABC-HRP method (Figure 1), the latter method was used in the following experiments.

ABC-HRP method gives background in the liver

Endogenous peroxidase and biotin

Peroxidase activity results in decomposition of H_2O_2 and is a common property of several enzymes including catalases, which are abundant in the liver tissue. The protocol used (Table 1) was adequate to efficiently block endogenous peroxidase in the liver. Biotin is a vitamin and a coenzyme found in a wide variety of tissues. The liver is a tissue particularly rich in this compound. Thus, endogenous biotin can bind to the avidin from the ABC complex, resulting in background. In the liver tissue, some background due to endogenous biotin was always observed. Because prolonging the avidin/biotin blocking incubations (Table 1) for 30 min or 1 h did not lead to a decrease in the background level, this blocking step was followed according to the manufacturer's indications, i.e., for 15 min each. The level of background varied between patients (not shown).

Biotinylated anti-mouse antibodies give "false" positive cells

The secondary biotinylated goat anti-mouse antibody cross-reacted with some cells present both in tonsils and in liver. However, in tonsils only occasional 1-2 "false" positive cells per section were observed, while in liver tissue the number of these cells could reach up to 13 positive. This could be due to binding of the Fc region of the secondary antibody to Fc receptors, which are present on macrophages, granulocytes or B and some T cells. To attempt to block this putative binding, sections were incubated with 2 % of normal goat serum for 15 min, 30 min or 1 h, previously to the addition of the secondary antibody (Table 1, step 4). However, prolonging the time of incubation was not sufficient to block the binding of the secondary antibody.

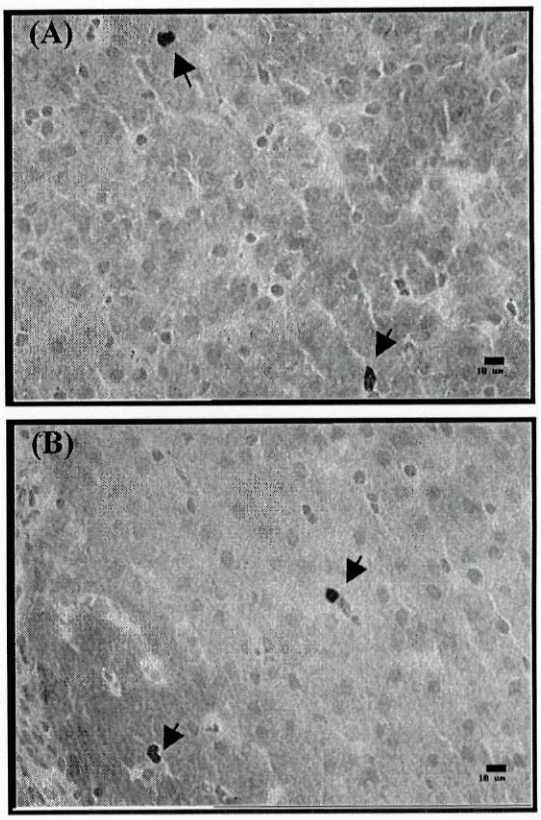


Figure 2 Liver section from a patient with hepatitis C virus (HCV) demonstrating that "false" positive cells (arrows) were obtained when goat $F(ab')_2$ fragments anti-mouse (A) or donkey $F(ab')_2$ fragments anti-mouse (B) were used. Original magnification ×250.

Non-specific staining due to binding of Fc region of an antibody to Fc receptors in the tissue can be avoided by using $F(ab')_2$ fragments, instead of the whole IgG molecules. Therefore, biotinylated goat $F(ab')_2$ fragments anti-mouse, adsorbed against human serum, were used as secondary antibody (Table 1, step 5). Several dilutions (1:500, 1:1000, 1:2500, 1:5000) were tested (primary antibody was omitted). Even at dilution 1:5000, "false" positive cells were still observed, indicating that this secondary antibody was cross-reacting with some cells present in the liver tissue (Figure 2A).

A secondary antibody produced in another host (donkey) was also tested (biotinylated donkey $F(ab')_2$ fragments anti-mouse) as described in the previous paragraph. However, this secondary antibody gave also "false" positive cells (Figure 2B).

In situ hybridisation

Activated PBMC were positively stained when antisense probes specific for TNF-α and IFN-γ were used, and were negatively stained when the sense probes were used. Figure 3A shows a representative staining of TNF-α positive cell in activated PBMC and Figure 3B the respective

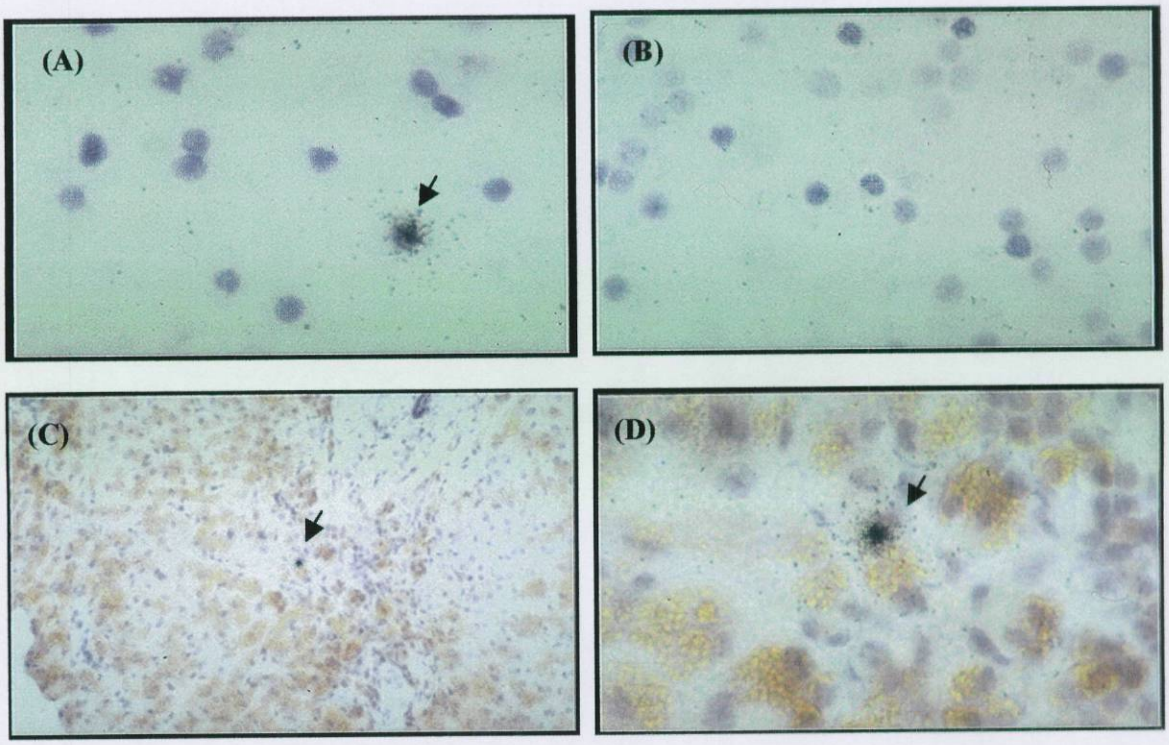


Figure 3 Demonstration by *in situ* hybridisation of TNF-α mRNA expressing cells in activated PBMC (A and B) and in liver tissue (C and D). A shows a TNF-α mRNA positive cell (arrow) obtained when ^{35}S-labelled antisense probes were used. No positive cells were observed (B) when the sense probes were used. In C a TNF-α mRNA positive cell is observed in portal tract of a patient with hereditary haemochromatosis. D shows the same cell as in C at a higher magnification. The original magnification is ×250 in A and B, ×100 in C and ×400 in D.

negative control, demonstrating that the sense probes did not bind to the cells. Figure 3C shows a TNF-α expressing cell in a liver section from a patient with HH (patient no. 6, in Table 2), and Figure 3D shows the same cell at a higher magnification. All liver sections of the alcoholic patients, two of the HH patients with fibrosis or cirrhosis, three of the controls and the tonsil section presented high levels of background with the sense probes. This background was mainly on the sections, and not on the glass slide, indicating that the probes were binding non-specifically on the sections. Thus, most of the biopsies were excluded, and only those with few or none "false" positive cells in the negative controls are shown in Table 2.

Table 2 TNF-α and IFN-γ producing cells in liver tissue determined by situ hybridisation

Patients	Age (years)	Fibrosis on biospy*	Sense probes†	anti-TNF-α probes†	TNF-α$^+$ cells‡	anti-IFN-γ probes†	IFN-γ$^+$ cells‡
HH							
1	30	1	0	1	**1**	7	**7**
2	36	1	3	2	**0**	0	**0**
3	51	1	3	1	**0**	1	**0**
4	51	1	0	0	**0**	2	**2**
5	53	1	2	1	**0**	0	**0**
6	69	2	1	5	**4**	2	**1**
Controls							
1	43	1	0	0	**0**	5	**5**
2	45	1	5	0	**0**	2	**0**

Note: all subjects were male. * 1 = no fibrosis; 2 = fibrosis; † number of positive cells per section; ‡ number of TNF-α or IFN-γ mRNA expressing cells per section (when fewer positive cells were obtained with the antisense probes, than with the sense probes, the number of positive cells per whole section was considered zero, see material and methods).

DISCUSSION

Several problems appeared when we tried to analyse the presence of cytokine producing cells in the liver tissue by immunohistochemistry. The APAAP method revealed to be less sensitive than the ABC-HRP method according with another earlier report (Hsu et al., 1981). Hence, in the subsequent experiments the later technique was used, which should confer certain advantages compared with the other previously described methods (Sander et al., 1991; Dolhain et al., 1993 Andersson et al., 1994). As reported before, staining of human tonsils from a patient with recurrent tonsillitis, was clearly positive for both TNF-α and IFN-γ producing cells (Andersson et

al., 1994). However, in the liver some difficulties occurred. Background due to endogenous peroxidase was completely eliminated with the protocol used, while that due to endogenous biotin was eliminated for the majority of the patients. The most disturbing obstacle was the cross-reactivity of all secondary antibodies tested with cells present in the liver tissue. Nevertheless, this was not the case in the tonsil tissue, where only 1-2 "false" positive cells were observed in the negative control, in contrast to more than 25 positive cells observed with antibodies against TNF-α or IFN-γ. Thus, local cytokine producing cells in the liver was then evaluated using the *in situ* hybridisation technique. This time, once again, the positive control (stimulated PBMC) showed a positive cell staining when the antisense probes were used, but no staining with the sense probes (negative control). However, in tissue sections (liver and tonsils) the sense probes gave "false" positive staining in the majority of the sections. Therefore, only in few patients the number of positive cells could be estimated. As shown in Table 2, very few positive cells (almost equal to the background level) were observed in liver sections from HH patients or controls. This might be due to the relative low grade of inflammation, detected by haematoxylin and eosin staining, in HH and in normal liver tissue, compared to the tissues studied earlier where cytokine producing cells were found, such as in chronic viral hepatitis (Gonzalez-Amaro et al., 1994), chronic liver diseases (Hussain et al., 1994), inflammatory tonsillitis (Andersson et al., 1994), colitis due to shigellosis (Raqib et al., 1995), active rheumatoid arthritis (Ulfgren et al., 1995), inflammatory myopathies (Lundberg et al., 1997) and advanced atherosclerotic plaques (Frostegard et al., 1999). In fact, the observation that HH patients had low number of $CD8^+$ cells in the liver (chapter 3), in contrast with what happens in patients with HCV, could be an explanation for the difficulty in detecting cytokine producing cells in the former. Thus, more sensitive methods, such as reverse transcriptase polymerase reaction (RT-PCR) may be used to attain detectable cytokine levels in the liver. Finally, it was reported previously that there was an impaired release of TNF-α by PB monocytes-macrophages form patients with HH (Gordeuk et al., 1992). Thus, evaluation of potential cytokine producing cells in HH patients in peripheral blood (PB) lymphocytes using FACS analysis (Maino and Picker, 1998) could also give some insights about the immune status in this disease.

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APPENDIX II – DOUBLE IMMUNOHISTOCHEMICAL STAINING OF CD8 AND TIA-1 ANTIGENS: TECHNICAL CONSIDERATIONS

INTRODUCTION

In chapter 3, we showed that HH patients with higher number of $CD8^+$ cells in the liver lobuli had lower iron stores and normal histology. The CD8 marker has been shown to define either cells having cytotoxic or suppressor functions. Cytotoxic T lymphocytes (CTLs) can induce cell death in the target cells by different mechanisms (Apasov et al., 1993). One way consists in releasing dense-cored cytoplasmatic vesicles containing putative effector molecules such as perforin and serine proteases, following target cell recognition (Golstein et al., 1991). After inserting into target cell membranes, perforin polymerises to form non-specific ion channels. *In vitro* these channels appear to be sufficient to induce cell lysis. *In vivo* these proteins seem to function by inducing the target cells' own suicide program leading to apoptosis, a programmed form of cell death, which serves to eliminate excessive, damaged or dangerous cells from the organism, without promoting inflammation. Central to this autolytic pathway is the activation of an endogenous endonuclease that results in the degradation of target cell DNA into integer multiples of a 200 bp nucleosome-sized monomers. It has been shown that the granule-associated protein TIA-1 is restrictively expressed in CTLs and natural killer cells, that its expression is increased upon lymphocyte activation and that it is able to induce apoptosis in permeabilised target cells (Andersson et al., 1990; Tian et al., 1991).

HH is associated with excess of iron deposition in hepatocytes. In experimental models, it is clear that iron overload leads to increased lipid peroxidation. Signals of oxidative stress, such as increased hepatic malondialdehyde (MDA)-protein adducts and oxidative modified serum proteins, have been shown to be presented in the liver of untreated HH patients (Bacon et al., 1997). One possible function for the $CD8^+$ cells observed in the liver of patients with HH could be the induction of apoptosis in hepatocytes showing signals of oxidative stress.

To determine whether $CD8^+$ cells with the phenotype of cytotoxicity were present *in situ*, a double immunohistochemistry staining technique was developed.

METHODS

As decribed in chapter 3. See Table 1, for double immunohistochemical protocol.

Table 1 Protocol for immunoenzyme double staining

Step	Regents	Time
1.	0.3 % H_2O_2, in TBS	30 min
2.	Avidin	15 min
	Biotin	15 min
3.	Normal rabbit serum (1:10)	10 min
4.	mAb1, unlabelled (anti-CD8)	overnight
5.	Rabbit anti-mouse	30 min
6.	Alkaline phosphatase-antialkaline phospatase complex (APAAP)	30 min
7.	Rabbit anti-mouse	10 min
8.	APAAP	10 min
9.	Normal mouse serum (1:5)	45 min
10.	TNT	30 min
11.	mAb2, biotinylated (anti-TIA-1)	overnight
12.	SA-HRP	30 min
13	BT	10 min
14.	SA-HRP	30 min
15.	Detection of AP activity (Vector Blue)	10 min
16.	Detection of HRP activity (AEC)	30 min

RESULTS

Since both antibodies anti-CD8 and anti-TIA-1 molecules were raised in the same host specie (mouse), one of the antibodies (anti-TIA-1) was biotinylated and detected without using a secondary antibody, in order to avoid cross-reactivity. The conjugation of biotin to a protein, e.g. IgG is a relatively mild chemical reaction and thus the antibodies may be biotinylated without loss of their antigen-binding reactivity. The avidin-biotin methods take advantage of the fact that the vitamin biotin (a coenzyme of decarboxylase) binds with an extraordinary affinity to the glycoprotein derived from the white egg, avidin or to streptavidin (Figure 1). Since biotin is a small molecule, the penetration ability of biotinylated antibodies is not decreased. Avidin as a tetramer with four binding sites for biotin conjugates can form a link with two or more biotin conjugates, e.g., biotinylated antibody or biotin-labelled peroxidase.

Detection of TIA-1 antigen using the immunoenzyme single staining: ABC-HRP versus TSA method

First, biotinylated mAb anti-TIA-1 was titrated using a modification of the ABC-HRP method (with omission of the secondary antibody). The tissue was incubated with different concentrations of biotinylated mAb anti-TIA-1 antibody, followed by the ABC-HRP complex, (complex of peroxidase-conjugated biotin and avidin). This complex, by virtue of the polyvalent nature of avidin, binds to the biotin-conjugated primary antibody (Figure 1A). This technique was below the threshold of detection, i.e., the sensitivity of this method was not enough to perform

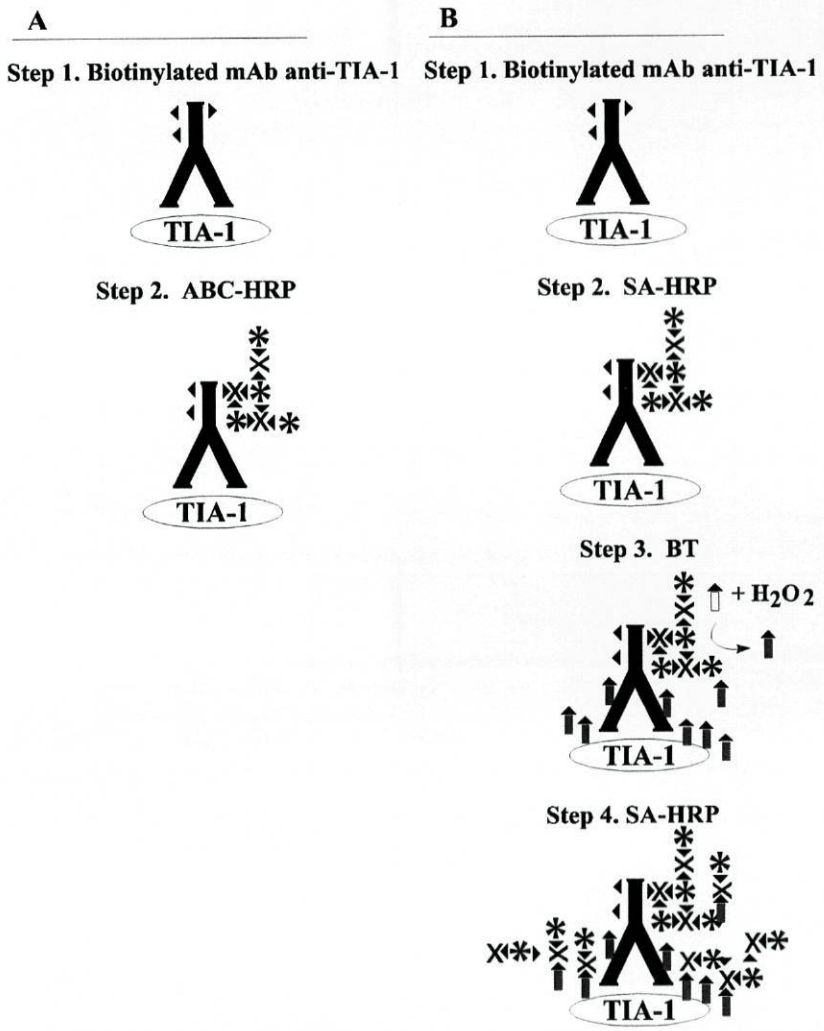


Figure 1 (A) Immunohistochemistry based on avidin-biotin interactions. Each HRP (asterisks) carries several biotin molecules (black triangles) which enables to form ABC complex with avidin molecules (X). Since some binding sites remain free, avidin of the ABC complex can be bound to biotinylated primary antibody (Y). (B) Tyramide signal amplification enables an enhancement of immunohistochemical signal; biotin (black triangles) attached to the primary antibody (Y) (Step 1) is recognised by streptavidin (X) couple to biotin-labelled-HRP (asterisks) (Step 2). At the beginning of the reaction, hydrogen peroxide (H_2O_2) and biotinylated tyramide, BT (white rectangles) are added (Step 3). Radicalised BT (grey rectangles) are attached to proteins found in the vicinity to the antigenic site (Step 3). Amplification is achieved through a second addition of HRP-labelled streptavidin molecules that bind to free biotin sites (Step 4).

immunohistochemistry staining (this antibody worked well with the APAAP method, which is a more sensitive technique since it includes the additional step of a secondary antibody, data not shown). Thus, in the next approach an amplification system, "tyramide signal amplification" (TSA) was used (Mokry, 1996). The TSA technology is based on the peroxidase catalysed deposition of multiple copies of biotinylated phenolic compound (in our case tyramide) on the tissue or cell preparation surfaces followed by a secondary reaction with streptavidin-HRP (Figure

1B). In the presence of hydrogen peroxide (H_2O_2), HRP catalyses the condensation of the phenolic compound, by a free radical mechanism. Radicalised phenols are covalently bound to electron rich moieties of surrounding proteins, i.e., on antibodies of detection system or the nearest tissue proteins. When such a phenolic compound is biotinylated, extra biotin molecules are introduced at the antigenic sites. The subsequent reaction with enzyme labelled streptavidin results in the coupling of additional enzymes. This method was shown to enhance the signal of the stainings up to 1000 fold. With this method a clear positive signal was observed and the best dilution (1:1000) of the mAb anti-TIA-1 to use in further experiments was determined.

Important points in the immunoenzyme double staining

Addition of the substrate of the first enzyme prior incubation with mAb2, hides the second antigen

Double staining (CD8 and TIA-1) was performed as described in the "Methods section" (chapter 3), with the following alterations. The blocking step with NMS (Table 1, Step 9) had not been introduced yet. The first substrate (Vector Blue) was added or omitted before incubating with the second primary antibody (anti-TIA-1). In this last case, the incubation with the first substrate was done only at the end followed by the DAB substrate (Table 1). Single staining for the TIA-1 molecule was performed in parallel (all reagents from step 3 to step 9 were substituted by TBS buffer, Table 1).

In the Table 2 one can see that when the substrate of the first enzyme was added prior the incubation of the mAb2 ("addition" protocol), less TIA-1 positive cells per mm^2 were counted in the double stained slide (96.6), compared to the single stained slide (160.0). Thus, the addition of the first substrate prior incubation of the second primary antibody seemed to block the access of this antibody to the intracellular antigen, TIA-1.

However, although the total number of TIA-1^+ cells per mm^2 counted in double stained slide (181.1) was more similar when the substrates were incubated in a final step, slightly more positive cells were counted compared to the single stained slide (160.0). In fact, as shown in the Table 2, slightly more TIA-1^+ cells were counted in the double stained slide than in the single stained slide (compare the two last columns in the Table 2, $P = 0.02$, paired t-test), in the majority of the patients (I, III, IV, V, VI and VII). In general, it appeared that some single $CD8^+$ cells were also being counted as TIA-1^+ cells. One explanation for this could be that the second primary antibody (mouse anti-TIA-1) was binding to the second antibody (rabbit anti-mouse IgG) that was free, i.e., not binding to the APAAP complex. To test this hypothesis, an experiment was made where this

putative cross-reactivity was blocked, by incubating the sections with normal mouse serum prior to addition of the second primary antibody (anti-TIA-1).

Table 2 Number of positive cells in the double and single stained slides per mm^2 in the liver lobuli

Protocol*	Patient	Diagnosis	Double staining†				Single staining†
			$CD8^+$	$TIA\text{-}1^+$	$CD8^+TIA\text{-}1^+$	Total$TIA\text{-}1^+$	$TIA\text{-}1^+$
Addition	I	HCV	33.6	62.4	34.2	96.6	160.0
Omission		HCV	73.6	72.6	108.5	181.1	
Omission	II	HH	14.1	27.2	4.5	31.7	32.3
Omission	III	HH	10.1	19.4	13.4	32.8	24.0
Omission	IV	HH	5.6	54.4	34.4	88.8	51.2
Omission	V	HH	19.7	27.0	5.0	32.0	31.4
Omission	VI	Control	2.4	113.3	54.1	167.4	86.1
Omission	VII	ALD	0.0	168.0	100.8	268.8	164.8

*Protocol: "Addition", when the first substrate was added immediately after incubation with the first enzyme; "Omission", when the first substrate incubation was omitted, and was only applied at the end of all the staining steps, just before the HRP substrate; † number of positive cells per mm^2 in the liver lobuli.

Blocking with NMS is necessary to prevent cross-reactivity

The following experiment was chosen to illustrate the importance of the blocking step with NMS, prior addition of mAb2. Sections were incubated without or with 3 different dilutions of NMS (1:20, 1:10 and 1:5) before applying the second primary antibody. All other steps were performed as shown the Table 1. As one can see in Figure 2, with increasing concentrations of blocking with NMS, the percentage of $CD8^+$ cells that were also positive for TIA-1 decreases (from 100 % to 66 %). Thus, this showed that in this case, without the blocking step about 30 % of $CD8^+$ cells were "false" double positive due to cross-reactivity.

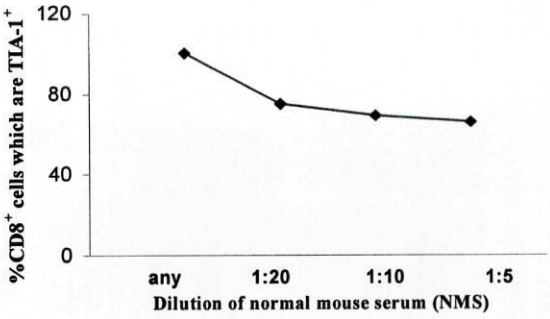


Figure 2. Graphic showing that the percentage of $CD8^+$ cells that were double stained (i.e. also $TIA\text{-}1^+$) decreased when a blocking step was introduced in the double staining protocol. Without blocking, all CD8 positive cells were also positive for TIA-1 molecule. However, blocking with increasing concentrations of normal mouse serum (NMS) revealed that in fact only about 70 % of the $CD8^+$ cells were also $TIA\text{-}1^+$.

Colour of the substrates

The visualisation of two antigens, which are in the same cell and therefore are almost overlapping antigens, needs an optimal colour combination. Three different combinations were tried and the development of AP activity in blue (Vector Blue)/HRP in red (AEC) was the one that showed the highest contrast between both basic colours (blue and red) and their mixed colour intermediate (purple). When AP (blue) and HRP (brown) were used, the co-expression displayed a "dark blue" colour, which was not always easy to distinguish from the single blue staining. When the AP (red) and HRP (brown) were used, the contrast was not very good. As referred in the "methods section" (chapter 3), the best time for substrate incubation was determined by observing the colour development under the microscope. In fact, the relative intensity of the two coloured precipitates is also an important factor in obtaining the best visualisation of the antigens.

DISCUSSION

Several double immunohistochemical stainings have been used in the past to co-localise expressed antigens. When the available antibodies against the antigens that we are interested to stain are raised in the same specie, a combination of an unlabelled and a biotinylated mAb has been previously described (van der Loos et al., 1988). Therefore, in our study the unlabelled mAb anti-CD8 was used in an indirect method (APAAP method, where a second antibody is used) and the biotinylated mAb anti-TIA-1 antigen was used that a direct method (TSA method, without using a secondary antibody). We have shown that there are three relevant points, which should be considered, namely the introduction of a blocking step with normal mouse serum, the importance of the colours' combination and the timing of substrates' addition. The protocol used in the immunoenzyme double staining (Table 1) takes these points into consideration. Since the ABC-HRP technique was not sensitive enough to stain the TIA-1 antigen, an enhancement step with biotinylated-tyramide was introduced. In this protocol, the concealing of the second antigen by substrate deposition was avoided by performing all the incubation steps before the colour development of both enzymes. In fact, it has been previously suggested that diaminobenzidine polymer product after developing HRP activity covered the second antigen (Sternberger and Joseph, 1979). Another important point in this protocol is the blocking step with NMS, which prevents a cross-reaction in step 5 and 11 (Table 1). This avoids the second primary antibody (mouse anti-TIA-1) to bind to the possible rabbit anti-mouse antibodies that were not bound to the APAAP complex. The colour combination of AP activity in blue and HRP activity in red showed the best contrast. This combination has also been previously used (Boorsma, 1984) in stained tissues where both epitopes to be detected were presented on packed morphological structures (e.g.

lymphocytes in infiltrates). Purple stained co-expression can be readily discriminated from both red and blue.

In summary, this multiple staining immunoenzyme technique was performed on tissue to detect antigens with almost overlapping location (i.e. in the same cell). Appropriate enzyme conjugates and their histochemical visualisation methods have been chosen in order to be able to detect these antigens clearly and selectively.

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