PATHOPHYSIOLOGICAL ASPECTS OF THE ACUTE PHASE RESPONSE AND THE ANAEMIA OF CHRONIC DISEASE

With a focus on iron metabolism

PATHOPHYSIOLOGISCHE ASPECTEN VAN DE ACUTE PHASE RESPONS EN DE ANAEMIE GEASSOCIEERD MET CHRONISCHE ZIEKTEN

In het bijzonder het ijzermetabolisme

R.A. Feelders

Pathophysiological aspects of the acute phase response and the anaemia of chronic disease, with a focus on iron metabolism

This thesis was prepared at the Department of Chemical Pathology, Erasmus University, Rotterdam, the Departments of Rheumatology and Surgical Oncology, Dr. Daniel den Hoed Clinic, Rotterdam and the Department of Autoimmune Diseases, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

Front page: Bryce Canyon National Park: rocks with a typical red-brown colour due to a high iron content

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Voor Jeroen

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List of abbreviations

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4-STf	4-sialotransferrin
ACD	anaemia of chronic disease
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
APP	acute phase protein
APRF	acute phase response factor
BFU-E	burst forming units-erythroid
CFU-E	colony forming units-erythroid
CIE	crossed immunoelectrophoresis
Con A	concanavalin A
CRH	corticotropin-releasing factor
CRP	C-reactive protein
ELISA	enzyme linked immunosorbent assay
EPO	erythropoietin
ESR	erythrocyte sedimentation rate
ETU	erythron transferrin uptake
Hb	haemoglobin
HPA	hypothalamic-pituitary-adrenal
HPT	hypothalamic-pituitary-thyroid
HSTf	highly sialylated transferrins
ID	iron deficiency
IDA	iron deficiency anaemia
IEF	isoelectric focusing
IFNγ	interferon γ
IL	interleukin
IL-1RA	IL-1 receptor antagonist
ILP	isolated limb perfusion
IRE	iron responsive element
IRP	iron regulatory protein
JAK	Janus-activated kinases
Ka	association constant
LMW	low molecular weight
NF-IL-6	nuclear factor-interleukin-6
NO	nitric oxyde
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
MHC	major histocompatibility
MPS	mononuclear phagocyte sysytem
LSTf	low sialylated transferrins
RA	rheumatoid arthritis
RBC	red blood cell
r-HuEPO	recombinant human erythropoietin
r-HuIFNγ	recombinant human interferon γ
r-HuTNF	recombinant human tumor necrosis factor α
RIA	radio-immuno assay
rT3	reverse triiodothyronine

SAA	serum amyloid A
STAT	signal transducers and activators of transcription
sTNF-R	soluble tumor necrosis factor receptor
sTfR	soluble transferrin receptor
T2	diiodothyronine
T3	triiodothyronine
T4	thyroxine
TBG	thyroxine-binding globulin
TGFβ	transforming growth factor-β
Th	T-helper lymphocyte
TNFα	tumor necrosis factor α
TfR	transferrin receptor
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone

Chapter 1

INTRODUCTION

Chapter 1: Introduction

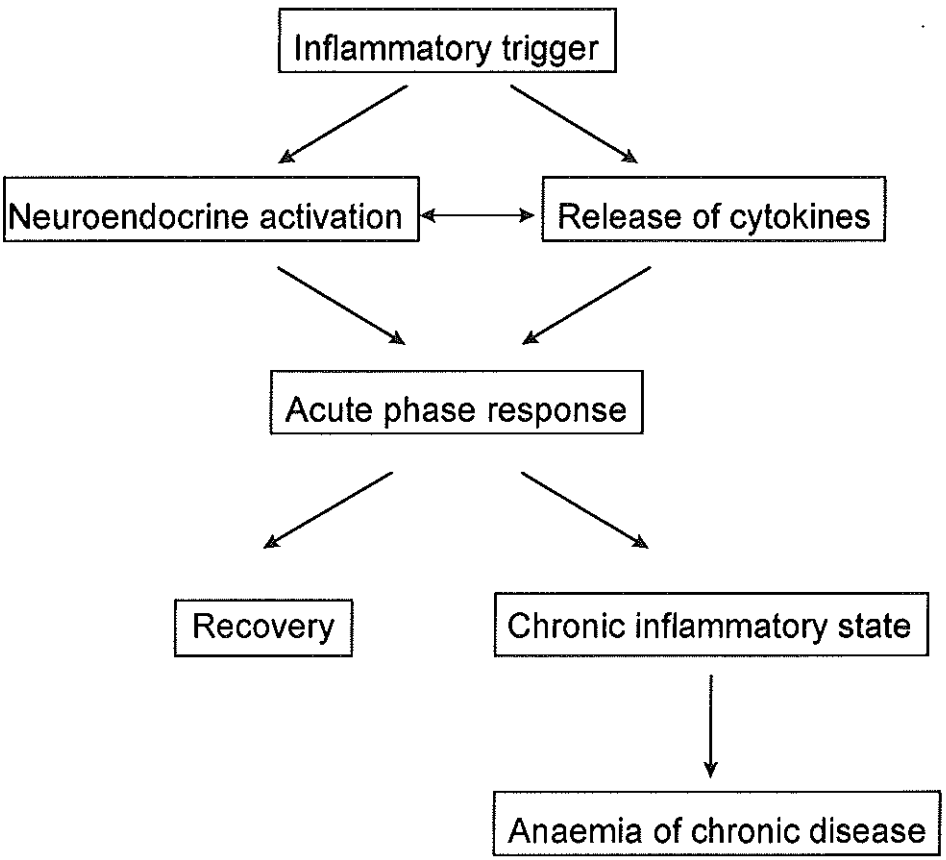


Figure 1: Host responses to an inflammatory stimulus.

1.1 General introduction

The acute phase response refers to a coordinated series of reactions in response to a variety of stimuli and involves all major body systems. This generalized host response is characterized by fever, changes in local vascular permeability, modulation of hepatic protein synthesis and systemic metabolic changes. Regulation of the acute phase response is thought to be mediated primarily by cytokines. Furthermore, complex interactions exist between the activated cytokine network and neuroendocrine systems, providing additional regulatory pathways.

The acute phase response may be transient and recover within a few days or, in case of persistence of the initiating stimulus, may be converted to a chronic phase of inflammation (Fig.1). Transition of the acute phase response to the chronic inflammatory state is frequently accompanied by the development of anaemia, defined as the anaemia of chronic disease [ACD]. ACD is associated with chronic infectious, inflammatory and neoplastic disorders and is a common cause of anaemia. The pathogenesis of ACD is complex and not entirely understood. Factors thought to be involved include inhibition of erythropoiesis, disturbance of iron metabolism and an impaired erythropoietin response to the anaemia. The induction of ACD is related to continuation of the inflammatory response, which might imply that ACD is an immunologically mediated phenomenon. Indeed, evidence has been obtained that pro-inflammatory cytokines are involved in the formerly mentioned pathophysiological mechanisms, although many of their postulated effects remain speculative.

The purpose of the present thesis is to obtain more insight in the regulation of the acute phase response and the pathogenesis of ACD with respect to the role of cytokines, endocrine systems and iron metabolism. In this perspective, the acute phase response and cytokine network are described in more detail in this chapter. Subsequently, current concepts of iron metabolism and the effects of inflammation are reviewed as well as pathogenetic, diagnostic and treatment aspects of ACD. Finally, the aims of the thesis wil be formulated.

1.2 Acute phase response

1.2.1 General aspects

Disturbance of homeostasis can be induced by numerous mechanisms and is usually followed by a coordinated sequence of physiological changes collectively known as the acute phase response. Challenges which initiate the acute phase response include trauma, major surgery, burn injury, ischaemic necrosis, infection, malignant growth and inflammatory disorders. The primary function of the acute phase response is to maintain or restore homeostasis through prevention of ongoing tissue damage, neutralization of the inflammatory agent and facilitation of repair processes. The acute phase response may include local reactions, involving changes in vascular tone and permeability and leucocyte migration into affected tissue, and/or systemic reactions, depending on nature, duration and severity of the inflammatory stimulus (1-3).

The spectrum of systemic reactions to inflammation is summarized in Table 1 and includes: (a) induction of fever and anorexia; (b) altered synthesis and glycosylation of plasma proteins; (c) changes in haematopoiesis; (d) altered serum levels of trace elements, e.g. hypoferraemia; (e) complement activation; (f) activation of the clotting cascade; (g) modulation of various endocrine systems, e.g. activation of the cortisol axis; (h) changes in substrate metabolism: increased gluconeogenesis and lipolysis, enhanced catabolism of skeletal muscle protein with subsequent amino acid transport to the liver and increased energy expenditure (1-7).

This pattern of physiological changes is similar in a variety of pathological conditions, pointing to the existence of common regulatory pathways. The induction and coordination of the acute phase response is thought to be regulated primarily by cytokines which are operative in a complex network. Proinflammatory cytokines, such as tumor necrosis factor α and interleukin-1, induce a cascade of other mediators which also participate in the regulation of the acute phase response (3,8-10). In addition, both stimulatory and inhibitory interactions exist between the cytokine network and endocrine systems, e.g. the cortisol axis, which modulate the acute phase response (11). The activated cytokine network is involved in many disease states which might explain the uniformity of alterations in the acute phase response irrespective of the underlying etiology.

Section 1.2 will deal with the following aspects of the acute phase response: cytokine network, acute phase protein synthesis and glycosylation and endocrine changes, in particular with respect to cortisol and thyroid hormone metabolism.

Table 1. Characteristics of the systemic acute phase response.

Organ/system	Change
Central nervous system	Fever Anorexia
Liver	Acute phase proteins: Δ synthesis Δ glycosylation
Haematopoiesis	Leukocytosis, thrombocytosis, anaemia
Trace elements	Hypoferraemia, hypozincaemia
Complement system	Synthesis↑, catabolism ↑
Clotting cascade	Synthesis↑, catabolism ↑
Endocrine systems	Activation of the adrenal axis, inhibition of the thyroid axis, growth hormone↑, insulin↑, glucagon↑, catecholamines↑
Substrate metabolism	Gluconeogenesis↑, lipolysis↑, proteolysis skeletal muscle↑, energy expenditure↑

1.2.2 Cytokines

Cytokines are hormone-like polypeptides, produced by white blood cells and a variety of other cell types, which exert their pleiotropic activities in both a paracrine, autocrine and endocrine mode and play a major role in inflammatory responses (8). Cytokines modulate various cellular processess such as gene expression and cell growth via binding to high-affinity cytokine receptors which are present on many cell types. The production of cytokines is often transient and once released, the circulating half-life is usually short. The proinflammatory cytokines tumor necrosis factor α, interleukin-1, interleukin-6 and interferon γ will be dicussed separately.

1.2.2.1 Characteristics of proinflammatory cytokines

Tumor necrosis factor α

As early as the end of the 19th century it was observed that intercurrent bacterial infections can reduce the size of a malignant tumor. The endogenous factor capable of inducing tumor lysis was isolated in the mid-1980s and termed tumor necrosis factor α [TNF] (12). TNF is an important proximal mediator of the endogenous inflammatory response and plays a central role in host defense functions (9). TNF is predominantly produced by activated macrophages/ monocytes and to a lesser extent by other cell types such as lymphocytes and vascular smooth muscle cells (13-15). TNF is initially synthesized as a 26 kDa precursor protein, which is processed to a 17 kDa mature protein via proteolytic cleavage by a microsomal metalloprotease named TNF α converting enzyme (16,17). TNF gene transcription is initiated by a variety of stimuli (Table 2) such as endotoxin, exotoxins and cytokines like interleukin-1, but also by TNF itself (17-21). The circulating half-life of TNF is short, approximately 14-18 minutes, and organs possibly involved in TNF degradation are liver, spleen, skin, lung and kidney (22,23).

TNF exerts a broad spectrum of biological activities which are summarized in Table 3. The biological effects of TNF are initiated by binding of TNF to specific cell surface receptors. Two distinct TNF receptors have been identified with a molecular mass of 55 [TNF-R55] and 75 kDa [TNF-R75] respectively, which are expressed on the majority of cell types (24). There is no homology between the intracellular domains of TNF-R55 and TNF-R75 suggesting that both receptors act through different signaling pathways. Indeed, signals from TNF-R55 and TNF-R75 are not redundant and each receptor is thought to be responsible for distinct TNF activities. Effects of TNF transmitted by TNF-R55 include cytotoxicity, endothelial cell activation and induction of the transcriptional factor NfκB, whereas TNF-R75 signals TNF-mediated proliferation of primary thymocytes and T-cells (24). Shedding of the extracellular

Table 2. Characteristics of proinflammatory cytokines.

	TNF	**IL-1β**	**IL-6**	**IFNγ**
Mol. mass	17 kDa	17.5 kDa	21-28 kDa	34 kDa
Chromosome	6	2	7	12
Predominant cell source	Macrophages/ monocytes	Macrophages/ monocytes	Endothelial cells fibroblasts, T-/ B-cells, monocytes	T-cells, natural killer cells
Receptor	TNF-R55, -R75	Type I and II	IL6Rα-gp130	IFNγ-R
Production / by	Endo/exotoxins viral particles TNF, IL1, IL2, IFNγ	Endo/exotoxins viral particles TNF, IL1, IL2, IFNγ	Endo/exotoxins viral particles TNF, IL1, IL2, PDGF, IFNγ	Antigen/MHCII IL1, IL2, TNF
Production / by	IL4, IL6, IL10, IL13, cortisol	IL4, IL6, IL10, IL13, cortisol	Cortisol	IL4, IL10, cortisol

Characteristics of the cytokines tumor necrosis factor α, interleukin-1β, interleukin-6 and interferon γ: molecular mass, gene localization, main cell source, cytokine receptors and factors which stimulate and inhibite the production of the particular cytokine. MHC II, major histocompatibility [MHC] class II antigens; PDGF, platelet derived growth factor.

domains of TNF-R55 and TNF-R75 results in circulating soluble TNF receptors [sTNF-R]. Elevated sTNF-R55 and -R75 levels have been observed in a variety of clinical conditions and, in contrast to TNF, both sTNF-R's may circulate for prolonged periods in acute and chronic inflammatory states (25). The exact kinetics of sTNF-R's in the acute phase response, however, are not known in detail (see aims of the study *1.5.5*). Both sTNF-R's are able to bind TNF and may attenuate TNF bioactivity, providing a mechanism to counteract biological effects of TNF (25).

Elevated plasma TNF levels have been demonstrated in a variety of disorders such as bacterial and parasitic infections, AIDS, cancer and rheumatoid arthritis (26-29). It must be emphasized, however, that plasma TNF levels do not necessarily reflect tissue TNF levels due to rapid TNF clearance form the circulation. TNF is thought to be involved in the development of septic shock and the adult respiratory distress syndrome and may thus be a potential harmful mediator. On the other hand, studies in experimental animals have shown that TNF has an essential role in antibacterial resistance (30). TNF has also been implicated in the pathogenesis of other disease states including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and cachexia associated with AIDS and malignancy (29,31,32).

Interleukin-1

The term interleukin-1 [IL-1] refers to two proinflammatory cytokines, IL-1α and IL-1β, and one cytokine inhibitor, IL-1 receptor antagonist [IL-1RA]. Although IL-1α and IL-1β are products of distinct genes, both cytokines are structurally related, bind to the same receptor and share biological activities (33). Both IL-1α and IL-1β are synthesized as a precursor protein of 31 kDa, whereas the molecular mass of the mature forms is 17.5 kDa. Pro-IL-1α remains in the cytosol or is transported to the cell membrane and expressed at the cell surface. Membrane-associated IL-1α may exert biological effects in a paracrine or autocrine mode. Processing of IL-1β involves cleaving of the precursor protein to the 17.5 kDa active form by the IL-1β converting enzyme. Subsequently, IL-1β is released into the circulation (33,34).

IL-1 is like TNF predominantly synthesized by activated macrophages/monocytes in response to similar stimuli (Table 2) (33). Two IL-1 receptors have been identified. IL-1 receptor type I is expressed by many cell types and signal transduction is mediated via phosphorylation of protein kinases. IL-1 receptor type II is a non-signaling receptor and is found on neutrophils, monocytes and B lymphocytes. The extracellular domains of type I and II IL-1 receptors are found as soluble receptors in the circulation, similar to soluble TNF receptors (33,35).

IL-1 is a pleiotropic cytokine and exerts similar proinflammatory, immunostimulatory, haemodynamic and metabolic effects as TNF (Table 3). IL-1 not only shares these biological

Table 3. Biological effects of TNF and IL-1.

Organ/cell type	**Effect**
Immune system	Activation of neutrophils, T- and B-cells, induction of cytokines and prostaglandins, cytotoxic effects to infected and tumor cells
Liver	Induction of acute phase proteins
Endothelial cells	Expression of adhesion molecules, release of nitric oxyde and procoagulatory substances
Fibroblasts	Proliferation
Bone/cartilage	Resorption
Haematopoiesis	Inhibition of erythropoiesis
Central nervous system	Fever, anorexia
Endocrine systems	Induction of stress hormones (cortisol, catecholamines etc.)
Intermediary metabolism	Proteolysis, lipolysis, glucose turnover↑, energy expenditure↑, weight loss

activities with TNF, but also acts synergistically with TNF, although both cytokines are structurally different and act through different receptor systems. This synergism between IL-1 and TNF may be explained by common post-receptor signal transduction pathways (9,33). Plasma IL-1 levels are elevated in various disease states such as sepsis, burn injury and rheumatoid arthritis (36-38), but are relatively low compared to TNF and IL-6 levels which is attributed to binding of IL-1 to soluble type II IL-1 receptors and plasma proteins like α2-macroglobulin and complement factors (33). Nonetheless, in view of its proinflammatory properties and its predominant local production in inflammation, IL-1 has been implicated as an important pathogenetic factor in many diseases (33). The effects of IL-1 are counteracted by IL-1RA via binding of IL-1RA to IL-1 receptors without triggering of signal transduction. Plasma IL-1RA levels are elevated in many clinical disorders and correlate with disease severity (39).

Interleukin-6

Interleukin-6 [IL-6] is a multifunctional cytokine which has a central role in the regulation of the acute phase reaction, the immune response and haematopoiesis. In contrast to plasma levels of TNF and IL-1, which are not always elevated in inflammatory states, plasma IL-6 levels are usually, at least transiently, increased in the acute phase response. IL-6 is therefore thought to act as a systemic endocrine mediator, transmitting a long-distance message and thereby serving as a systemic alarm signal in response to tissue injury (40,41). IL-6 gene expression can be induced in a variety of cell types (Table 2) and is stimulated by cytokines, predominantly TNF and IL-1, and micobial products (40-42). IL-6 synthesis is inhibited by glucocorticoids (42,43).

The IL-6 receptor is expressed by both lymphoid and non-lymphoid cells and consists of an 80 kDa IL-6 binding protein, termed IL-6Rα, and a 130 kDa glycoprotein [gp130], a signal transducing subunit. Binding of IL-6 triggers an interaction between IL-6Rα and gp130 which is followed by activation of tyrosine kinases, named Janus-activated kinases [JAK] which phosphorylate cytoplasmic proteins termed signal transducers and activators of transcription [STAT]. Phosphorylated STATs translocate to the nucleus where they interact with IL-6 responsive genes (44). IL-6Rα is also shed as soluble IL-6 receptor, however, in contrast to soluble TNF and IL-1 receptors sIL-6Rα acts as an IL-6 agonist (45). IL-6 is member of the IL-6-like cytokine family which includes leukemia inhibitory factor, interleukin-11, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1. These cytokines have a similar helical structure and share the gp130 subunit in their receptors. The functional redundancy of IL-6 related cytokines is therefore, at least in part, explained by a common signal transduction pathway (44). IL-6 has multiple biological effects, which are summarized in Table 4 (41,46).

Table 4. Biological effects of IL-6.

Organ/cell system	Effect
Immune system	T- and B-cell differentiation↑ Synthesis of immunoglobulins↑ Myelomonocytic differentiation↑ TNF and IL-1 synthesis↓
Haematopoiesis	Haematopoietic stem cell differentiation↑ Megakaryocytosis↑
Liver	Regulation of acute phase protein synthesis and glycosylation
Central nervous system	Fever
Endocrine systems	Multiple effects, e.g. activation of cortisol axis
Miscellaneous	Tumor cell growth↑, e.g. myeloma cells

Interferon γ

Interferon γ [IFNγ] is member of the interferon family which includes IFNα, IFNβ and IFNγ. IFNα and IFNβ can be produced by any nuclear cell and have an important funtion in host defense against virus infections. IFNγ, in contrast, is exclusively produced by T cells and natural killer cells (Table 2) and has a key role in the activation of immune reactions and is therefore involved in host resistance to a broad spectrum of microbial challenges (47,48).

IFNγ is predominantly produced by the T-helper 1 [Th1] subclass of $CD4^+$ T-cells. IFNγ production is initiated after Th1 cell activation via antigen presentation by macrophages in the context of major histocompatibility [MHC] class II antigens. Interaction between antigen/MHCII and the T-cell receptor-CD3 complex results, in the presence of a co-stimulatory signal such as IL-1, in T-cell proliferation and the production of IFNγ and IL-2. IL-2, in turn,

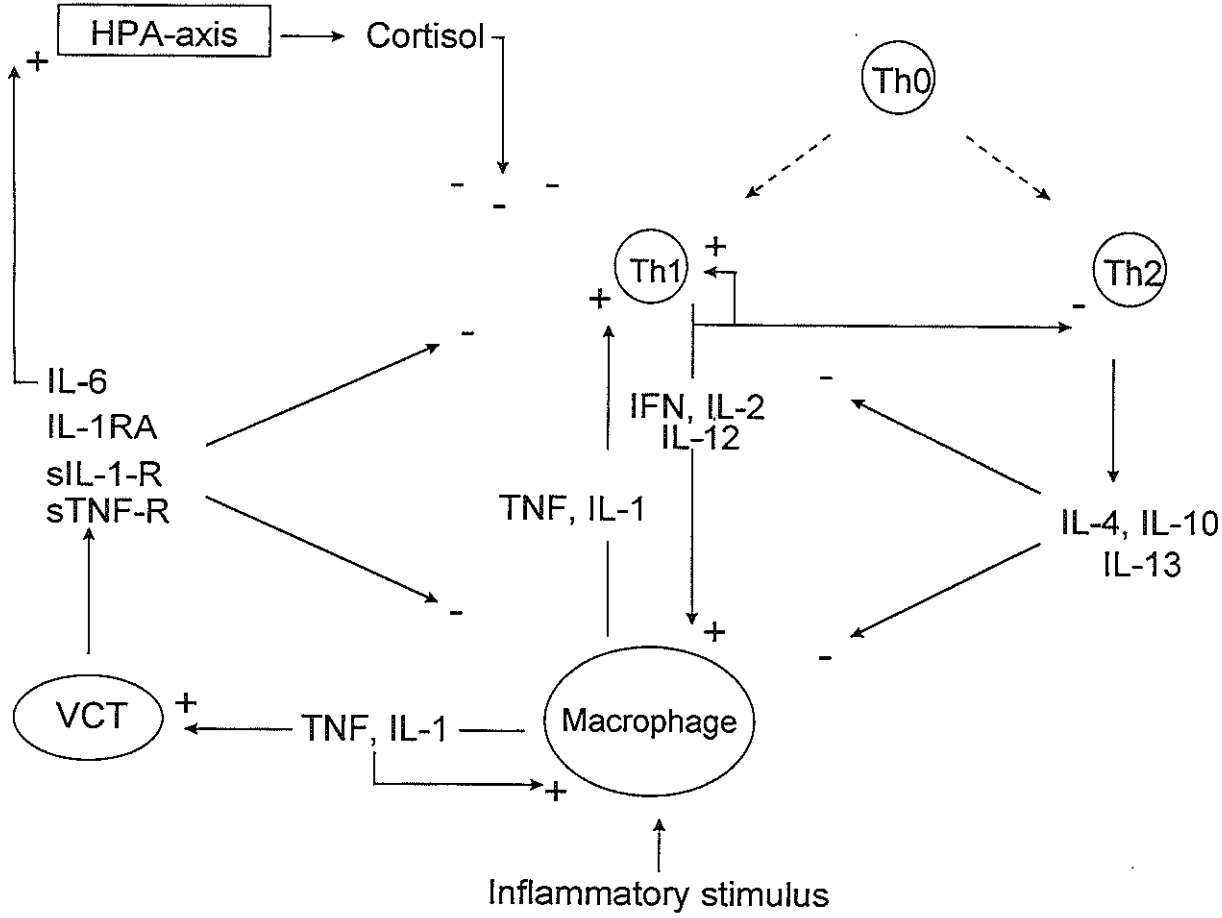
synergizes with IL-1 in the induction of IFNγ synthesis (47-49).

IFNγ acts through a specific cell surface receptor and IFNγ receptor expression on monocytes is upregulated by TNF and IL-1 (48). After binding of IFNγ to its receptor, signal transduction is mediated via activation of JAK-STAT pathways (48). IFNγ has many immunomodulatory effects, e.g. induction of MHC class I and II antigen expression and stimulation of T- and B-cell proliferation. One of the major functions of IFNγ, however, is activation of macrophages and regulation of macrophage effector functions, e.g. production and release of cytokines, nitric oxyde [NO] and reactive oxygen intermediates, expression of Fc receptors and phagocytosis, intracellular killing of microorganisms and anti-tumor activity (48). Elevated serum IFNγ concentrations have been found in patients with infectious and autoimmune disorders (50,51).

1.2.2.2 Cytokine network

Cytokines are operating in a complex network and their ultimate biological effects are the resultant of synergistic and antagonistic interactions between involved cytokines at the level of cytokine synthesis, cytokine receptor expression and cytokine bioactivity. The cytokine cascade is usually triggered by macrophage activation with a concomitant release of TNF and IL-1, which act as proximal mediators in the cytokine network. TNF and IL-1 stimulate their own but also each others synthesis and receptor expression. TNF and IL-1 induce a second wave of cytokines such as IL-2, a T cell growth factor (54), IL-6, an important mediator of the acute phase response and IL-8, a chemotactic and activating factor for neutrophils (55). Interaction of the activated macrophage with Th1 cells results in IL-2 and IFNγ production (see **IFNγ**). IFNγ, in turn, is a major activator of macrophages and stimulates the release of TNF and IL-1, thereby creating a positive feedback loop (46-48). IL-2 is a major stimulus of T-cell proliferation and TNF and IL-1 augment T-cell responses not only by increasing IL-2 production but also by induction of IL-2 receptor expression (9,33). Thus, several amplifying steps in the activated cytokine cascade potentiate the inflammatory response (Fig. 2).

Figure 2. Schematic representation of the cytokine network in response to an inflammatory stimulus. Stimulatory (+) and inhibitory (-) interactions between immunocompetent cells and secreted cytokines, soluble cytokine receptors and cytokine receptor antagonists are described in 1.2.2.2. Abbreviations: Th: T-helper lymphocyte; VCT: various cell types (neutrophils, monocytes etc.); HPA-axis, hypothalamus-pituitary-adrenal axis; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon gamma; sTNF-R, soluble TNF receptors; sIL-1-R, soluble IL-1 receptors; IL-1RA, IL-1 receptor antagonist.


HPA-axis
Cortisol
Th0
Th1
Th2
IL-6
IL-1RA
sIL-1-R
sTNF-R
IFN, IL-2
IL-12
TNF, IL-1
IL-4, IL-10
IL-13
VCT
TNF, IL-1
Macrophage
Inflammatory stimulus

The inflammatory response is antagonized by several counterregulatory mechanisms including: (a) negative feedback loops, e.g. inhibition of TNF and IL-1 production by IL-6 (56) and suppression of TNF, IL-1, IL-2, IL-6 and IFNγ synthesis by glucocorticoids (33,43,57); (b) attenuating cytokine bioactivity by soluble cytokine receptors, e.g. soluble TNF and IL-1 receptors (25,33), and cytokine receptor antagonists, e.g. IL-1RA (39); (c) production of antiinflammatory cytokines, e.g. IL-4 and IL-10 (Fig. 2). T-helper cells can be subdivided in Th1, Th2 and Th0 cells, based on the pattern of cytokine production (58). Th1 cells are involved in cell mediated inflammatory reactions and produce IFNγ, IL-2 and IL-12. Th2 cells regulate humoral responses and secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Th0 cells produce multiple cytokines and can differentiate into either Th1 or Th2 cells. Th1 cytokines inhibit the synthesis of the Th2 cytokine pattern and vice versa. The Th2 cytokines IL-4, IL-10 and IL-13 are considered as antiinflammatory cytokines and suppress the production of TNF, IL-1 and the Th1 cytokines IFNγ and IL-2 (58). In addition, IL-4 stimulates the release of soluble IL-1 receptors and IL-1RA (33,59).

1.2.3 Acute phase proteins

1.2.3.1 Synthesis

One of the major characteristics of the acute phase response is the modulation of hepatic protein synthesis resulting in a rapid increase or appearance of a distinct subset of plasma proteins, known as acute phase proteins [APP]. Plasma proteins which concentrations decrease during the acute phase response, e.g. albumin and transferrin, are designated as negative APP. The time course of APP concentrations shows a sequential pattern in the acute phase response with changes in APP levels occuring at different rates and to different degrees. For instance, C-reactive protein [CRP] and serum amyloid A [SAA] rise early in the acute phase response, i.e. within 24 hour, and can increase to 1000-fold over normal values. This is followed by a second wave of APP including APP like α1-antitrypsin, fibrinogen and complement factors, which increase modestly, i.e. between 50 % and several-fold over initial levels (Fig. 3). APP have several important functions in inflammatory and tissue repair processes: (a) facilitation of opsonization and phagocytosis, e.g. CRP, secretory phospholipase A_2 and complement factors; (b) antiproteolytic effects, e.g. α1-antitrypsin; (c) antioxidant activity, e.g. haptoglobin, haemopexin; (d) haemostasis, e.g. fibrinogen (1-3,60).

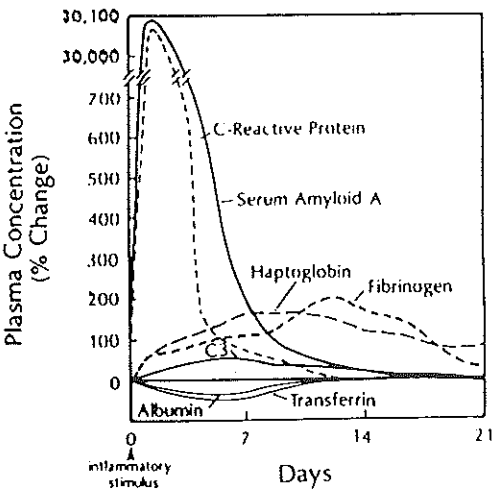


Figure 3.
Sequential acute phase protein patterns following an inflammatory stimulus. From ref. 2.

Cytokines modulate protein synthetic rate of APP as has been shown by *in vitro* studies with rat hepatocytes, human hepatoma cell lines and human hepatocytes (2,3,61-64). APP can be subdivided into two groups according to the cytokine subsets which regulate APP synthesis *in vitro* (Table 5). Type 1 APP are CRP, SAA, complement factor C3 and α1-acid glycoprotein and are induced by IL-1 type cytokines which comprise IL1 α and β and TNF α and β. Type 2 APP are fibrinogen, haptoglobin, α1-antitrypsin and ceruloplasmin and are regulated by IL-6 type cytokines which include next to IL-6, leukemia inhibitory factor, interleukin-11, oncostatin M and ciliary neurotrophic factor (3,62-68). Generally, IL-6 type cytokines synergize with IL-1 type cytokines in the regulation of type 1 APP but not vice versa (3,62). Indeed, IL-6 is considered as the major regulator of APP synthesis and stimulates the full spectrum of APP in primary human hepatocytes *in vitro* (63). The synthesis of the negative APP albumin and transferrin is inhibited by both IL-1 and IL-6 type cytokines *in vitro* (63,64). Glucocorticoids potentiate the effects of IL-1 and IL-6 type cytokines on APP synthesis (69). Other factors which may modulate cytokine action on APP synthesis include insulin, thyroxin and transforming growth factor-β [TGFβ] (3).

Table 5. Classification and regulation of acute phase proteins.

APP classification	APP	Regulation
Positive APP		
Type 1 APP	C-reactive protein	*IL-1 type cytokines*:
	serum amyloid A	IL-1 α and β
	α1-acid-glycoprotein	TNF α and β
	complement C3	*Potentiated by*:
		IL-6 type cytokines
		glucocorticoids
Type 2 APP	α1-antitrypsin	*IL-6 type cytokines*:
	fibrinogen	IL-6, IL-11, LIF,
	haptoglobin	OSM, CNTF
	hemopexin	*Potentiated by*:
	ceruloplasmin	glucocorticoids
Negative APP	(pre-) albumin	*IL-1 and IL-6 type cytokines*
	transferrin	
	α2-HS glycoprotein	

Classification of APP's. Positive APP's are subdivided in type 1 and type 2 APP's according to the cytokine subsets which regulate the synthesis of these APP's in vitro. Abbreviations: APP, acute phase proteins; IL, interleukin; TNF, tumor necrosis factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor.

Cytokine-controlled APP synthesis is primarily mediated at transcriptional level (3,61-64,70-73), although regulation at translational and post-translational level has also been described, e.g. for CRP (74,75). Thus, regulation of APP synthesis is highly complex and involves cooperative action of several mediators which modulate APP synthesis at different levels. Little is known about the *in vivo* effects of cytokines on APP synthesis in humans. It is difficult, however, to extrapolate the *in vitro* effects of individual cytokines to the situation *in vivo*, as in the acute phase response hepatocytes are exposed to a sequence and/or combination of interacting cytokines rather than to a single cytokine. Moreover, *in vivo*, cytokines presumably operate at different concentrations than in the *in vitro* setting. One of the aims of this thesis is to examine the *in vivo* effects of cytokine administration on APP levels in humans (see *1.5.5*).

1.2.3.2 Glycosylation

The majority of APP, with the exception of CRP, SAA and albumin, are glycoproteins which contain one or more N-linked complex type oligosaccharides. This carbohydrate moiety can vary with respect to: (a) the degree of glycan branching, indicated by the existence of bi-, tri- and tetra-antennary glycans; (b) the terminal carbohydrate composition, e.g. variation in the number of sialic acid residues. As a result, each APP has a distinct microheterogeneity pattern, composed of a limited number of subfractions, which can be distinguished by means of electrophoretic techniques (76-78).

Acute and chronic inflammatory states, e.g. burn injury and rheumatoid arthritis, are associated with alterations in microheterogeneity patterns of various APP which are related to disease activity (79-84). Alterations in glycosylation may depend on disease stage. In acute inflammation the microheterogeneity patterns of α1-antitrypsin and α1-acid-glycoprotein show a shift towards isoforms with biantennary glycans, whereas in chronic inflammation the subfractions with tri- and tetraantennary glycans predominate (83,84). Therefore, glycosylation of APP may be differentially regulated in acute and chronic inflammation. Apart from the degree of glycan branching, changes may occur in the terminal carbohydrate composition as reflected by variations in glycan sialylation. For instance, in rheumatoid arthritis sialic acid content of α1-acid-glycoprotein is decreased (85) whereas transferrin sialylation is increased (86). The microheterogeneity of human transferrin in health and disease is described in detail in *1.3.2.1* and one of the questions addressed in this thesis is whether in rheumatoid arthritis changes in transferrin glycosylation are related to disease activity, cytokines and the presence of the anaemia of chronic disease (see *1.5.1*).

Cytokines have been postulated to modulate post-translational modification of APP. *In vitro* studies with human hepatoma cell lines and primary human hepatocytes have shown that

mediators like TGFβ, IFNγ, IL-1 and IL-6 and its related cytokines influence glycosylation patterns of several APP such as α1-antitrypsin, α1-acid-glycoprotein and ceruloplasmin (87-92). For instance, in primary human hepatocytes IL-1 and IL-6 increase the synthesis of α1-acid-glycoprotein, in particular the subfractions with biantennary glycans (89). *In vivo*, IL-6 exerts similar effects on the microheterogeneity pattern of α1-acid-glycoprotein in rats (89). No data are available on the *in vivo* effects of cytokines on APP glycosylation in humans.

Protein glycosylation occurs in the endoplasmic reticulum and Golgi apparatus and involves a sequential enzymatic proces. A precursor oligosaccharide is processed to N-linked glycans by glycosidases, glycosyltransferases and mannosidases. Subsequently, N-acetylglucosaminyl-transferases catalyze the formation of bi-, tri- or tetra-antennary structures which can be modified by galactosyl-, fucosyl- and sialyl-transferases (76,77). The regulatory mechanism of glycan synthesis has not been elucidated yet, but appears to be a highly coordinated process, despite the absence of an accurate template mechanism as operative in nucleic acid and protein synthesis (76). Cytokines may influence post-translational modification of APP by modulating the synthesis or activity of glycosylation enzymes (93,94).

What are the consequences of alterations in glycan composition of APP in the acute phase response ? The exact physiological functions of the carbohydrate moiety of plasma proteins are only partly understood. Nonetheless, oligosaccharide side chains may affect physico-chemical properties and therefore biological activities of proteins (76,77). It has been demonstrated for several APP that their microheterogenous forms can differ in functional properties (95,96). Thus, modulaton of APP glycosylation, with a concomitant predominance of particular APP subfractions, may have a role in the fine tuning of APP functions in the acute phase response.

1.2.4 Endocrine changes in the acute phase response

1.2.4.1 General aspects

The systemic response to inflammation is associated with adaptive alterations in various endocrine axes (11). Recently, increasing evidence has become available concerning the existence of a complex interplay between immune and neuroendocrine systems (11,97). Regulation of endocrine functions is primarily mediated by the hypothalamic-pituitary axis. In the stress response, the hypothalamus integrates afferent signals from the central nervous system, but also from the immune system as transduced by cytokines, which is eventually followed by changes in hormonal responses (11,97). Cytokines modulate hypothalamic-pituitary function and major endocrine adaptations in the acute phase response are activation of

the hypothalamic-pituitary-adrenal axis and inhibition of the hypothalamic-pituitary-thyroid axis (4,5) [see below]. Other endocrine changes observed in inflammation and possibly mediated by cytokines are: inhibition of the hypothalamic-pituitary-gonadal axis and increased circulating levels of prolactin, growth hormone, vasopressin, catecholamines, insulin, glucagone and renin (11). The effects of cytokines on synthesis and secretion of these hormones, however, are complex and their exact role remains to be elucidated. For instance, both stimulatory and inhibitory effects of IL-1 and TNF have been reported on the secretion of prolactin and growth hormone (11).

The interactions between immune and neuroendocrine systems appear to be bidirectional, as endocrine hormones influence immune effector functions. For instance, *in vitro* studies have shown that growth hormone and prolactin act as growth factors for lymphocytes and that corticotropin-releasing hormone [CRH] has proinflammatory effects like T cell proliferation and cytokine release (97,98). Complexity of immuno-neuroendocrine interactions increases by the fact that cells of the immune system itself may synthesize hormones, such as prolactin and growth hormone production by lymphocytes (97). Conversely, cytokines are expressed in many endocrine glands, as has for instance been shown for TNF and IL-1 in hypothalamus, thyroid and adrenals (11).

1.2.4.2 Hypothalamic-pituitary-adrenal axis

Glucocorticoid hormone production is regulated by the hypothalamic-pituitary-adrenal [HPA] axis. CRH is secreted by the hypothalamus into the portal circulation and acts on the pituitary to elicit the release of adrenocorticotrophic hormone [ACTH]. ACTH then stimulates the production and release of glucocorticoids by the adrenal glands. Glucocorticoids, in turn, exert negative feedback effects on CRH and ACTH secretion. Activation of the HPA axis in the acute phase response is thought to be mediated by cytokines. TNF, IL-1, IL-2 and IL-6 stimulate ACTH release, both directly and indirectly via induction of CRH, the latter being important for early HPA axis activation (4,11,99). In addition, TNF, IL-1, IL-2 and IL-6 may also promote glucocorticoid production at the level of the adrenal cortex (100,101). In this thesis plasma cortisol levels are examined in relation to TNF and IL-6 profiles during the induction and recovery of the acute phase response (see *1.5.8*).

In inflammatory stress, activation of the HPA axis is an essential adaptive response. First, glucocorticoids have profound metabolic effects resulting in increased catabolism and concomitant substrate mobilization. Second, in view of their immunosuppressive properties, glucocorticoids play an important role in the counterregulation of the inflammatory response (see also *1.2.2.2*). Antiinflammatory effects of glucocorticoids include inhibition of adhesion

molecule expression, induction of eosinophil apoptosis, suppression of Th1 activity and inhibition of cytokine production, e.g. TNF, IL-1 and IL-6 (4,33,43).

1.2.4.3 Hypothalamic-pituitary-thyroid axis

The production of thyroxine [T4] and triiodothyronine [T3] by the thyroid is stimulated by thyroid-stimulating hormone [TSH], which is secreted by the pituitary. TSH release, in turn, increases in response to thyrotropin-releasing hormone [TRH], produced by the hypothalamus. T4 and T3 inhibit both TSH and TRH secretion by a negative feedback mechanism. T4 is considered as a prohormone of T3, which is the metabolically active hormone. Thyroidal T3 release accounts for 20 % of daily T3 production, whereas 80 % is formed via extra-thyroidal deiodination of T4. Peripheral conversion of T4 to T3 occurs predominantly in the liver and is mediated through deiodination of the outer ring of T4, catalyzed by an enzyme termed type I deiodinase. Inner ring deiodination converts T4 to reverse triiodothyronine [rT3], an inactive metabolite. Inner ring and outer ring deiodination of T3 and rT3 respectively results in the formation of diiodothyronine [T2]. In the circulation, thyroid hormones are bound to plasma proteins, in particular thyroxine-binding globulin [TBG] and, to a lesser extent, albumin and pre-albumin. The biological actions of thyroid hormones are thought to be mediated by the free fractions of T4 and T3, which exert their metabolic effects after cellular uptake via receptor-mediated endocytosis (102).

The systemic response to inflammation in both acute and chronic conditions is frequently accompanied by major alterations in thyroid hormone metabolism, defined as the euthyroid sick syndrome. This syndrome is characterized by low plasma T3 concentrations and increased levels of rT3 (5,103). T4 levels are at variance, but fall with increasing severity of the underlying disease (104). TSH values are usually normal, although elevated or reduced TSH levels can also be found (105,106). It is not known whether the decrease in T3 levels serves a functional purpose in the acute phase response. It has been postulated, though, that the low T3 state is an adaptative mechanism to counteract excessive catabolism by reducing metabolic demands in nonessential tissues.

The pathogenesis of the euthyroid sick syndrome is multifactorial and incompletely understood. Inhibition of hepatic type I 5'-deiodinase activity is thought to result in a decreased T3 production rate with a concomitant reduced clearance of rT3. In addition, T4 availability for peripheral conversion to T3 might be decreased based on reduced T4 synthesis and inhibition of T4 uptake by hepatocytes (5,103). Cytokines have been shown to modulate thyroid hormone metabolism *in vitro* (107-109) and *in vivo* (110-112). For instance, *in vitro* TNF and IL-1 inhibit TSH and thyroid hormone release by pituitary and thyroid cells respectively

(108,109). Administration of recombinant TNF to heathy volunteers and recombinant IL-6 to cancer patients resulted in changes in thyroid hormones compatible with the euthyroid sick syndrome (111,112). TNF might therefore influence thyroid hormone metabolism either directly or indirectly via induction of IL-6.

In summary, in the acute phase response complex interactions exist between the cytokine network and endocrine systems, which may participate in the regulation of metabolic changes in the acute phase response, e.g. APP synthesis. Presumed relationships are, however, mainly based on *in vitro* and experimental animal studies. In this thesis the *in vivo* effects of TNF are examined on cortisol and thyroid hormone metabolism in the acute phase response (see *1.5.8*).

1.3 Iron metabolism in health and in inflammation

1.3.1 Biology of iron

Iron is an essential element for virtually every form of life as it plays a key role in a spectrum of biological processes including oxygen transport by haemoglobin, electron transport in the process of oxydative phosphorylation and enzyme functions, e.g. hydroxylases and ribonucleotide reductase (113). Iron has a molecular mass of 56 Da and occurs in two valence states, the divalent ferrous form [Fe^{2+}] and the trivalent ferric form [Fe^{3+}]. At neutral pH, iron is nearly insoluble as illustrated by the very low solubility product of $Fe(OH)_3$ of 4.10^{38} (114). Therefore, in physiological circumstances, iron is transported and processed by either iron binding proteins, e.g. transferrin and ferritin, or low molecular weight [LMW] compounds such as citrate, amino acids and ATP. Iron may, however, also be potential harmful as it, presumably in its free or LMW form, is able to catalyze the formation of oxygen radicals such as hydoxyl radicals via the Haber-Weiss reaction (115). Reactive oxygen species can damage structures like DNA, lipids and proteins and have been implicated in the pathogenesis of various disorders, e.g. rheumatoid arthritis (116).

The human body contains 3-5 g iron of which approximately 70 % is incorporated into haemoglobin (Table 6). Body iron is distributed over two pools: first, a functional compartment of haemoglobin-iron in the erythron; second, a storage compartment of ferritin- and haemosiderin-bound iron, located mainly in liver and mononuclear phagocyte system [MPS] (117). Iron fluxes between functional and storage sites rely on transport by transferrin. The amount of transferrin-bound iron is approximately 3 mg and ferrokinetic studies have shown that plasma iron turnover is about 0.7 mg/dL whole blood/day (118,119). The MPS plays a central role in iron homeostasis, processing haemoglobin from senescent erythrocytes (120).

Table 6. Iron distribution in the human body.

Compartment	Component	mg Iron/75 kg (male)
Functional compartment	Haemoglobin	2300
	Myoglobin	320
	Haem enzymes	80
	Non-haem enzymes	100
	Transferrin	4
Storage compartment	Ferritin	700
	Haemosiderin	300
Total		3800

In physiological conditions bone marrow iron requirements determine the equilibrium between iron storage and release by the MPS. Daily iron loss is 0.5 to 1.5 g and is normally met by compensatory intestinal uptake. Iron absorption occurs mainly in the duodenum and refers to a sequence of mucosal iron uptake, transcellular transport and iron transfer to the portal circulation. The exact mechanism and its regulation, however, have not been elucidated yet (121). Nonetheless, iron balance appears to be regulated at the level of iron absorption as the rate of intestinal iron uptake is related to the rate of erythropoiesis (122).

1.3.2 Iron binding proteins

1.3.2.1 Transferrin

Transferrin is a monomeric glycoprotein with a molecular mass of 80 kDa consisting of a polypeptide chain of 679 aminoacids and two N-linked complex type glycan chains (Fig. 4). The transferrin molecule comprises two homologous domains, the N-terminal and C-terminal-

domain, each containing one iron binding site (123,124). The carbohydrate moiety is attached to the C-terminal domain. The two iron binding regions act independent of each other and at a pH of 7.4 the affinity constants of the N- and C-site are 1 and 6 x 10^{22} M^{-1} respectively (125).

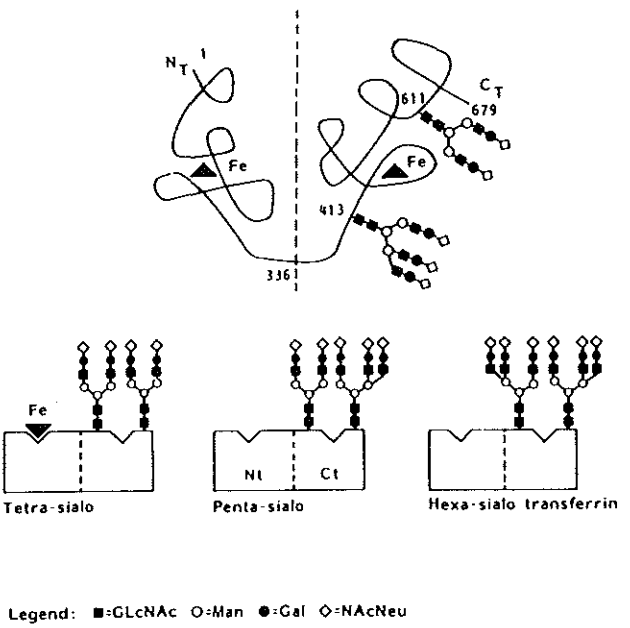


Figure 4. Schematic representation of the human transferrin molecule. Both the N- and C-terminal domain contain one iron binding site. The carbohydrate moiety is attached to the C-terminal domain and can vary with respect to the degree of branching and sialylation. From ref. 131.

The main function of transferrin is to transport iron from storage sites to target tissues. In addition, transferrin may serve as a growth factor and may play a role in non-specific antimicrobial defense (126). Transferrin is predominantly synthesized by hepatocytes, but can also be produced by other cell types such as Sertoli cells and brain capillary endothelial cells (126). Transferrin synthesis may be regulated at both transcriptional and translational level. Transcriptional regulation is thought to be mediated by a regulatory sequence, a so called iron responsive element, located in the 5'-untranslated region of the transferrin gene. Serum transferrin concentrations are increased during iron deficiency and pregnancy and decreased in haemochromatosis and inflammation (126,127).

Heterogeneity of serum transferrin is based on the following three determinants which influence its electrophoretic behaviour. (a) *Genetic polymorphism.* As a result of variations in the amino acid composition of the polypeptide chain, multiple transferrin variants can be detected by electrophoresis. The most common phenotype (more than 95 % in European populations) has been designated TfC. TfC itself consist of several subtypes which have been identified by isoelectric focusing [IEF] (128). (b) *Iron content.* By IEF, four different transferrin forms can be separated with respect to iron content: apotransferrin, monoferric transferrin with iron bound at the N- and C-terminal domain respectively, and diferric transferrin with both binding sites occupied by iron (126). (c) *Glycosylation.* General aspects concerning plasma protein glycosylation are described in *1.2.3.2.* The two N-linked complex type glycans of transferrin may vary with respect to the degree of branching and terminal carbohydrate composition, i.e. sialic acid content. By Concanavalin A [Con A] affinity chromatography 3 transferrin subfractions can be separated based on glycan branching. The distribution in healthy individuals is: bi-bi-antennary glycans 76 %, bi-tri-antennary glycans 20 % and tri-tri-antennary glycans 4 % (129,130). Transferrin subfractions can also be separated according to sialic acid content. The two glycans can be completely sialylated or partially desialylated, which may also be accompanied by absence of galactose and N-acetyl glucosamine residues. Consequently, 9 transferrin isotypes can be distinguished by IEF, based on differences in isoelectric points (pH range 5 to 6), determined by the number of sialic acid residues (Fig. 5).

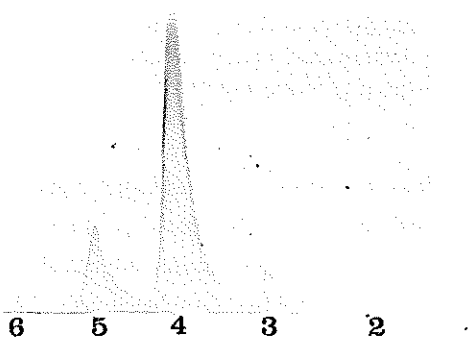


Figure 5. Transferrin microheterogeneity pattern of a healthy individual as analyzed by crossed immunoisoelectric focusing. The indices beneath the pattern indicate the number of sialic acids attached to the N-linked glycans of corresponding isotypes.

The predominant fraction is the tetra-sialo variant, whereas the 0-, 1- and 8-sialo fractions are usually undetectable. Inter- and intra-individual variations of microheterogeneity patterns are

minimal (131). The spectrum of transferrin sialo-isotypes correlates with the 3 Con A separated fractions, as IEF of these Con A fractions has revealed that bi-bi-, bi-tri- and tri-tri-antennary fractions consist predominantly of 4-, 5- and 6-sialo transferrins respectively (129,130). Therefore, analysis of transferrin microheterogeneity by IEF can be used to assess protein glycosylation with respect to both glycan sialylation and, at least in part, glycan branching.

Alterations in the transferrin microheterogeneity pattern can be observed in several physiological and pathological conditions. During pregnancy, the concentrations of the 4-sialo, but predominantly the highly sialylated fractions increase, whereas the desialylated fractions remain constant. This increase in highly sialylated transferrin isotypes parallels the increase in transplacental iron transport (131). Similar changes, although less pronounced, are observed in women using oral contraceptives and in iron deficiency anaemia (131,132). Alcohol abuse is associated with an increase in transferrin variants with a low sialic acid content (131). In various diseases such as rheumatoid arthritis, cancer and haemochromatosis a shift is observed towards highly sialylated transferrin fractions (86). These changes occur presumably in the context of altered protein glycosylation in the acute phase response (see *1.2.3.2*). The question whether modulation of transferrin glycosylation in pregnancy and disease is of functional significance in iron homeostasis has not been answered yet.

1.3.2.2 Ferritin

Ferritin is the primary iron storage protein and consists of an apoprotein shell enclosing a core of iron, stored in the form of ferric hydroxy-phosphate. The protein shell consists of 24 subunits of 2 types, termed the heavy [H] and light [L] subunit with a molecular mass of 21 and 19 kDa respectively (133). The composition of the protein shell can vary with respect to the proportions of H- and L-subunits. H-rich ferritin is expressed in the heart and erythrocytes, whereas L-rich ferritin is predominantly produced by liver and spleen (134). These isoferritins may differ in functional properties. The rate of iron uptake by H-rich ferritin is higher than by L-rich ferritin. Iron content, however, is usually higher in L-rich ferritin. It has therefore been proposed that H-rich ferritin enables the cell to respond rapidly to an increase in intracellular iron and that L-rich ferritin serves as a long-term storage protein (134-136). Iron release by ferritin might be mediated by acidification in lysosomes (136), the exact mechanism, however, has not been clarified yet. Ferritin synthesis is primarily regulated at translational level and is discussed in *1.3.3.*

Ferritin is mainly located in the intracellular compartment and circulating ferritin concentrations are low in physiological circumstances. In contrast to intracellular ferritin, serum

ferritin appears to be glycosylated (137). Ferritin receptors have been identified at various tissues, e.g. liver and erythrocytes, and circulating ferritin might be cleared via receptor mediated endocytosis (138). In the absence of inflammation, serum ferritin reflects body iron stores, with 1 µg/l serum ferritin corresponding to 8-10 mg storage iron (139). Serum ferritin is therefore an important diagnostic tool in the detection of iron deficiency. Elevated serum ferritin levels are found in iron overload and inflammation (see *1.3.5*).

1.3.2.3 Transferrin receptor

Iron transport across the cell membrane is facilitated through interaction of transferrin with the transferrin receptor [TfR]. The human TfR is a transmembrane dimeric glycoprotein composed of 2 identical 95 kDa subunits, linked by disulfide bonds. Each monomer consists of a phosphorylated cytoplasmic N-terminal domain, a hydrophobic transmembrane segment and a large extracellular segment which includes the C-terminal domain to which 3 oligosaccharide chains are attached (Fig. 6). Each subunit is able to bind one transferrin molecule (140-142). At a pH of 7.4, the affinity of the TfR is higher for diferric transferrin as compared to monoferric- or apotransferrin (143). The carbohydrate moiety of the TfR may influence transferrin binding, since nonglycosylated TfR have a lower affinity for transferrin. In addition, TfR glycosylation may be important for dimerization and migration of TfR to the cell surface (144). TfR from different tissues appear to be identical, both structurally and immunologically (145).

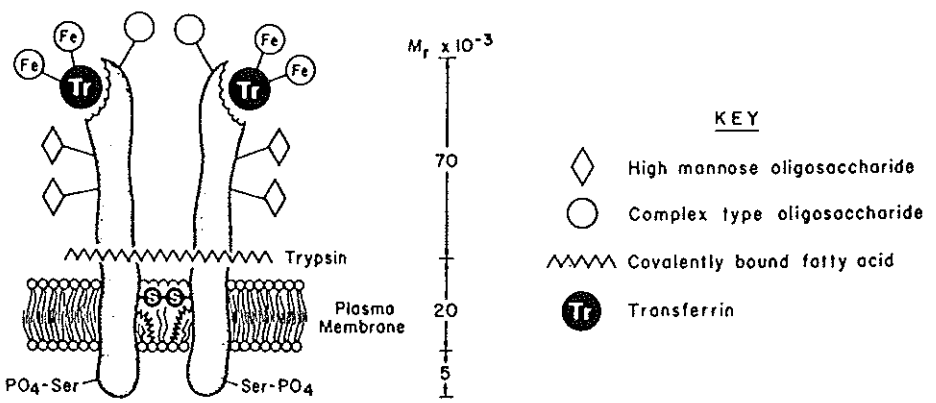


Figure 6. Schematic representation of the human transferrin receptor molecule.

Cellular TfR distribution involves TfR expressed at the cell surface and an intracellular TfR pool, which presumably consists of TfR participating in the endocytic cycle (see *1.3.3*) and TfR located in storage pools (146,147). The number of surface TfR is influenced by cell differentiation, cell proliferation and cellular iron demands (146-149). Upregulation of TfR expression may result from an increased de novo TfR synthesis and/or from mobilization of TfR from storage pools (see *1.3.3* for regulation). TfR are predominantly expressed by maturing cells of the erythroid lineage, which have high iron requirements for haeme synthesis, and syncytiotrophoblasts (150,151). However, TfR can be identified on nearly every cell type, in particular proliferating cells of both malignant and nonmalignant origin (148,152,153).

1.3.3 Cellular iron uptake and iron homeostasis

The predominant mode of cellular iron uptake is receptor mediated endocytosis of diferric transferrin, and generally a close correlation exists between iron uptake and the number of TfR (150). The endocytic cycle is a sequential process which starts with binding of transferrin by the TfR (154). Subsequently, receptor-ligand complexes are concentrated in clathrin coated pits and internalized through formation of coated vesicles (155,156). Intracellularly, the pH within the endocytic vesicle is lowered by an ATP-dependent proton pump to about 5.5, which is essential for dissociation of iron from transferrin (157,158). In addition to acidification, a conformational change of transferrin after its binding to the TfR may change its affinity for iron (159). Finally, iron release may be facilitated by LMW chelating agents like ATP (160). Once released, iron is transported over the vesicle membrane via an unknown carrier mechanism, possibly after reduction of iron (161). In the cytoplasm, iron is bound to LMW compounds, e.g. citrate, oxalate and ATP. Not explained, however, is the mechanism and its coordination by which iron is directed to its final destinations, i.e. storage into ferritin or incorporation into haemoglobin or iron-dependent enzymes. ATP, as iron carrier may have an important role in intracellular iron transport (161). Apotransferrin remains bound to the TfR at the low pH in the endocytic vesicle and the complex recycles to the cell surface where, at neutral pH, apotransferrin dissociates from the TfR (162).

Other mechanisms postulated to be involved in cellular iron uptake are: (a) endocytosis of transferrin by the asialoglycoprotein receptor, present on hepatocytes (163); (b) iron release from transferrin at the cell surface, mediated by a TfR associated NADH reductase, followed by iron transfer via a membrane-bound iron carrier (164); (c) iron delivery by other iron containing proteins, e.g. ferritin (165). Whether these alternative pathways significantly contribute to cellular iron acquisition remains to be established.

Control of cellular iron homeostasis is mediated via regulation of ferritin and TfR synthesis at post-transcriptional level. This involves interaction of cytoplasmic RNA binding proteins, known as iron regulatory proteins [IRP], with mRNA stem loop structures defined as iron responsive elements [IRE] (Fig 7). IRE are located in the 5' and 3' untranslated regions of mRNAs encoding for ferritin and TfR respectively (166,167). The RNA binding acitivity of IRP is coordinated by the intracellular iron concentration, which influences the Fe-S cluster of IRP with a concomitant modulation of protein conformation (166). In cellular iron deficiency, but also in the presence of NO, IRP binds with high affinity to IRE. As a result ferritin translation is repressed whereas TfR mRNA is stabilized, facilitating TfR synthesis and iron uptake. Conversely, when cellular iron is in excess IRP acts as an aconitase with low RNA binding activity, allowing translation of ferritin mRNA. In contrast, degradation of TfR mRNA is accelerated, limiting TfR synthesis and iron uptake (166-170). This regulatory mechanism enables the cell to coordinate iron uptake, utilization and storage according to iron availability and iron requirements.

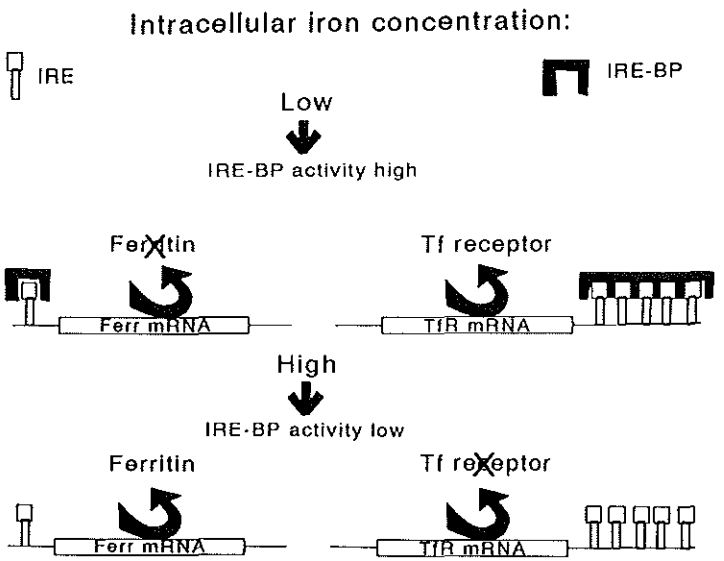


Figure 7.
Regulation of cellular iron homeostasis at post-transcriptional level by interaction of iron responsive element binding protein [IRE-BP] with iron responsive elements [IRE] located on ferritin and transferrin receptor mRNAs. Courtesy Dr. W. van Gelder.

1.3.4 Serum transferrin receptor

The TfR is found in a soluble form in human serum (171,172). This serum TfR [sTfR] is a truncated form of the intact TfR and has been identified as a monomeric fragment of the extracellular domain with a molecular mass of 85 kDa (173). The sTfR is generated by proteolytic cleavage of the TfR between Arg-100 and Leu-101 (173). In the endocytic cycle, a minor amount of endocytosed TfR is processed in a different mode. In this pathway, the endosome with TfR-ligand complexes matures to a multivesicular endosome via intravesicular outpouching of the endosome membrane. This multivesicular endosome contains multiple vesicles, termed exosomes, which are externalized by exocytosis. TfR proteolysis is mediated by a membrane associated serine protease, which is predominantly located at the exosome surface. The cell surface may be an alternative site for TfR truncation. Proteolytic cleavage of the TfR at the exosome surface occurs intracellularly, presumably within the multivesicular endosome. Subsequently, sTfR are released by exocytosis and are complexed to transferrin in the circulation (174,175). No data are available on the mechanism of clearance of sTfR.

Several studies have shown that the sTfR concentration is proportional to tissue TfR levels, i.e. erythroid TfR content, since 80 % of total TfR body mass is expressed by the erythroid colony forming unit and late erythroid precursors (176-179). This relationship between membrane and circulating TfR has been illustrated by measurement of erythron transferrin uptake [ETU: the amount of transferrin uptake by tissue TfR per unit time], a ferrokinetic parameter which is related to erythroid proliferation rate. A close correlation was found between sTfR levels and ETU in healthy individuals and in patients with various haematologic disorders (177,178). The number of erythroid surface TfR is determined by erythroid proliferation rate and cellular iron demands (see *1.3.2.3*). As sTfR levels reflect erythroid TfR turnover, the sTfR concentration is viewed as a quantitative measure of erythroid precursor cell mass and an index of tissue iron stores (176-178).

In healthy individuals, no age- or sex related differences in sTfR levels have been detected (172,177). Elevated sTfR levels are found in (see Table 7): (a) disorders with an increased erythroid proliferation, e.g. haemolytic anaemias and polycythemia vera; (b) iron deficiency anaemia [IDA] (176-180). In the assessment of body iron stores, sTfR levels appear to be a reliable marker of early tissue iron deficiency (179). Preliminary data suggest that sTfR may be useful in the differentiation of IDA from the anaemia of chronic disease (180) (see 1.4.3 and aims of the study *1.5.3*). Decreased sTfR levels are found in conditions accompanied by a hypoproliferative erythropoiesis, e.g. chronic renal failure and aplastic anaemia. In these disorders, sTfR levels can fall to approximately 50 % of control values, indicating that non-erythroid tissues contribute significantly to sTfR levels (172,177).

Table 7. Serum transferrin receptor [sTfR] levels in human disease.

sTfR concentration	Condition
Increased	
Erythroid proliferation ↑	Haemolysis
	Auto-immune haemolytic anaemia
	Hereditary spherocytosis
	β-Thalassemia/HbE
	Haemoglobin H disease Sickle cel anaemia
	Polycythemia vera
Tissue iron stores ↓	Iron deficiency anaemia
Normal to increased	Myelofibrosis Myelodysplastic syndrome Chronic lymphocytic leukemia
Normal	Haemochromatosis
	Acute and chronic myelogenous leukemia
	Most lymphoid malignancies
	Solid tumors
	Anaemia of chronic disease (?)
Decreased	Chronic renal failure
	Aplastic anaemia
	Post-bone marrow transplatation

1.3.5 Iron metabolism in the acute phase response

Inflammation is accompanied by profound changes in iron metabolism characterized by low serum iron concentrations associated with increased tissue iron stores. In addition, major alterations occur in serum levels of iron binding proteins reflected in low transferrin levels, decreased transferrin iron saturation and increased ferritin levels (181-183). In the chronic inflammatory state, prolonged hypoferraemia limits bone marrow iron availability and will ultimately impair erythropoiesis, thereby contributing to the development of the anaemia of chronic disease (see **1.4**). The changes in iron metabolism in the acute phase response are similar independent of the initiating stimulus, pointing to a possible role for cytokines. Indeed, cytokines modulate iron metabolism *in vivo* and *in vitro*, as will be discussed below.

1.3.5.1 Iron binding proteins in inflammation

Transferrin is considered as a negative APP and its serum concentration is usually decreased in inflammation and ACD (3,181-183). This might be mediated by cytokines like TNF, IL-1 and IL-6 which inhibit transferrin synthesis *in vitro* (see *1.2.3.1*). Apart from changes in the synthesis of transferrin, alterations may occur in its glycosylation pattern in the acute phase response as discussed in *1.3.2.1*. No data are available on the *in vivo* effects of cytokines on transferrin synthesis and glycosylation in humans (see aims of the study *1.5.7*).

Serum ferritin concentrations are elevated in a wide variety of infectious, inflammatory and malignant disorders (183). Ferritin is therefore thought to exibit acute phase behaviour and may even act as an early APP, analogous to CRP (184). The exact kinetics of ferritin in the acute phase response, however, have not been elucidated yet. Since the ratio between glycosylated and nonglycosylated ferritin forms (see *1.3.2.2*) remains constant in inflammation, elevated serum ferritin levels are thought to result from an increase in synthesis/secretion rather than from "leakage" of damaged cells (185). Indeed, in studies with experimental animals, evidence was found for both translational and transcriptional regulation of ferritin synthesis in inflammation (183). An increased apoferritin synthesis, presumably induced by cytokines, is likely to play a major role in the entrapment of iron in the acute phase response (see *1.3.5.2*).

Lactoferrin is an iron binding protein predominantly secreted by polymorphonuclear cells and high concentrations are found in inflammatory sites (186). In contrast to transferrin, lactoferrin is able to bind iron at an acid pH, frequently present in inflammatory areas. After uptake of the lactoferrin-iron complex by macrophages, iron can be incorporated into ferritin (187). Initially, it was proposed that inflammation-associated hypoferraemia relies on an increased lactoferrin secretion, as administration of lactoferrin to rats was followed by a decrease in serum iron levels (188). However, in this study supraphysiological amounts of lactoferrin were used (183).

In addition, in clinical conditions, hypoferraemia is not invariably associated with increased serum lactoferrin levels (184), although extravascular iron entrapment by lactoferrin can not be excluded. Nevertheless, lactoferrin is thought to play a minor role in the hypofaerremic response.

1.3.5.2 Mechanisms of hypoferraemia

The induction of hypoferraemia in the acute phase response is associated with an iron shift towards storage sites, reflected by an increased stainable iron content in macrophages of the MPS and hepatocytes (189,190). This iron redistribution results from iron retention by the storage compartment as illustrated by ferrokinetic studies in experimental animals. In rats with turpentine-induced inflammation, administration of ^{59}Fe-labelled non-viable erythrocytes is followed by an increased retention of radioiron by the MPS with a concomitant reduced plasma iron turnover (191). Similarly, hepatocytes of inflamed rats retain more iron compared to control rats after administration of ^{59}Fe-labelled haemoglobin-haptoglobin complexes or ^{59}Fe-labelled ferritin (190,192). Iron retention by the storage compartment is thought to be mediated primarily by an impaired iron release. In addition, iron influx into MPS and liver may concurrently be enhanced.

The MPS plays an important role in iron recirculation. After clearance of senescent erythrocytes and subsequent haeme catabolism, the obtained iron enters a labile intracellular iron pool and is either incorporated into ferritin or released to plasma transferrin, depending on bone marrow iron demand (120). Since this pathway represents 80 % of plasma iron turnover, it can be inferred that modulation of iron processing by macrophages in inflammation has profound effects on iron fluxes between MPS and bone marrow. Iron release by MPS and liver involves two distinct phases: an early phase representing iron release from the labile intracellular pool and a late phase reflecting release of iron stored in ferritin. Ferrokinetic studies in humans demonstrated that early MPS iron release is considerably reduced in inflammation. This decrease in iron release was associated with an increase in serum ferritin levels, which may indicate that labile iron is diverted to storage into ferritin (193). Indeed, in experimental animals the induction of hypoferraemia during inflammation correlates with an increased ferritin content in liver and spleen (191,194).

Theoretically, an impaired iron release might be based on: (a) a primary blockade of iron transfer across the cell membrane to the circulation, leading to an increase in labile iron with a subsequent iron-mediated stimulation of ferritin synthesis; (b) a primary induction of apoferritin synthesis, independent of iron, with a concomitant shift of labile iron into ferritin stores. In rats with turpentine-induced inflammation, an increased hepatic ferritin synthesis precedes the fall in

serum iron levels (191). This implies that a decreased tissue iron release results from entrapment of labile iron into an enhanced apoferritin pool, rather than from a primary membrane defect. It is not known, however, whether this mechanism is also operative in humans, which is one of the questions addressed in this thesis (see *1.5.6*). In addition to an impaired iron output, enhanced iron influx into storage sites may contribute to the induction of hypoferraemia. First, uptake of transferrin-bound iron by macrophages and hepatocytes obtained from inflammatory animals is significantly increased *in vitro* (195,196). Second, in inflammation erythrocyte survival may be shortened resulting in increased influx of haemoglobin-bound iron (182,183).

Cytokines are likely to be involved in the regulation of the hypoferraemic response. Administration of TNF, IL-1 or IL-6 to rodents induces a rapid decrease in serum iron levels, occurring within 3 to 6 hours (197-199). In rats, IL-6 reduces plasma half-life of ^{59}Fe-labelled transferrin, coinciding with an increase in liver and serum ferritin (199). *In vitro*, cytokines modulate iron handling by hepatocytes and macrophages. TNF, IL-1 and IL-6 induce TfR expression by hepatocytes with a concomitant increased iron uptake (199,200). The effects of cytokines on cells of the MPS are less clear. Macrophages obtained from TNF treated mice have an increased rate of transferrin uptake (201). However, iron uptake by rat Kupffer cells is not influenced by IL-6 (199) and in a human monocytic cell line TNF, IL-1 and IFNγ even decrease iron uptake (202). These conflicting results may be explained by differences in species and cell type and the use of different cytokine concentrations and incubation periods. *In vitro*, cytokines like TNF and IL-6 stimulate ferritin synthesis by various cell types such as hepatocytes, macrophages and fibroblasts (199,200,202-204). Cytokines may regulate ferritin synthesis independent of the intracellular iron concentration (202-204), supporting the concept of a primary increase in apoferritin synthesis in the acute phase response. The *in vivo* effects of cytokines on iron metabolism in humans, however, are presently unknown (see aims of the thesis *1.5.6* and *1.5.7*).

1.3.5.3 Functional aspects of hypoferraemia

The induction of hypoferraemia in inflammation may serve a functional purpose. First, it may be part of host-defense mechanisms by withholding iron from pathogenic microorganisms and tumor cells (205). Second, iron sequestration may protect host cells against formation of hydroxyl radicals, since generation of these toxic oxidants is catalyzed by free iron in inflammatory areas (206). Third, in macrophages low intracellular labile iron may promote cytokine responsiveness, cytokine production and immune effector functions, e.g. killing of intracellular microorganisms (207-209). In addition, a low iron environment may favour chemotaxis of neutrophils and their phagocytic function (210,211).

1.4 Anaemia of chronic disease

1.4.1 General aspects

Chronic infectious, inflammatory and malignant disorders are frequently accompanied by anaemia, defined as the anaemia of chronic disease [ACD] (181,182). ACD is characterized by hypoferraemia in the presence of normal to increased iron stores and does not include other causes of anaemia related to the underlying disease, e.g. haemolysis, bone marrow infiltration or bleeding. In addition, anaemia due to endocrine, hepatic or renal insufficiency is not designated as ACD. Cartwright proposed the following criteria for ACD: (a) decreased serum iron; (b) normal or decreased total iron binding capacity; (c) reduced transferrin iron saturation; (d) decreased number of sideroblasts and increased stainable iron in bone marrow (181).

ACD is, second to iron deficiency, the most common cause of anaemia in hospitalized patients (182). Cash and Sears analyzed a series of unselected hospitalized anaemic patients and 52 % of these patients were classified as having ACD (212). Interestingly, this study demonstrated that ACD can also be associated with diseases which do not fall in the "classical" categories, including chronic heart failure, ischaemic heart disease and deep leg vein thrombosis. Typically, ACD is a normocytic, normochromic anaemia which develops gradually within the first month of the underlying disease. The anaemia is usually mild, i.e. a fall in Hb to about 70 % of initial values, and has a non-progressive course (181,182). Many studies on ACD have been carried out in rheumatoid arthritis [RA] in which ACD frequently occurs (213).

1.4.2 Pathogenesis

The pathogenesis of ACD is complex and not entirely understood. The laboratory characteristics in ACD are similar irrespective of the underlying etiology, pointing to common pathogenetic mechanisms. Several studies show an inverse relationship between Hb levels and parameters of disease activity, e.g. ESR and CRP (213). In addition, a correlation between the degree of anaemia and macrophage activation was found in ACD accompanying infectious, inflammatory and neoplastic disorders (214). These observations suggest that ACD is initiated by the inflammatory response with the cytokine network as common denominator. Pathogenetic factors which are implicated in ACD include: (a) inhibition of erythropoiesis; (b) decreased erythroblast iron availability; (c) impaired erythropoietin response; (d) reduced erythrocyte survival (Table 8). These mechanisms and the possible role of cytokines are discussed separately.

Table 8. Proposed pathogenetic mechanisms in ACD and possible involved cytokines.

Pathogenetic factor	Possible involved cytokines
Inhibition of erythropoiesis	TNF, IL-1, IFNγ, IFNα, IFNβ, TGFβ
Decreased erythroblast iron availability	
- iron redistribution to storage sites	TNF, IL-1, IL-6, IFNγ
- decreased erythroid iron uptake	?
Impaired erythropoietin response	TNF, IL-1, IFNγ, TGFβ
Decreased bone marrow response to erythropoietin	TNF, IL-1, IFNγ
Reduced erythrocyte survival	TNF, IL-6

1.4.2.1 Inhibition of erythropoiesis

ACD is a hypoproliferative anaemia as illustrated by low reticulocyte counts for the degree of anaemia. In bone marrow of RA patients with ACD erythroid burst forming units [BFU-E] and colony forming units [CFU-E] were found to be decreased (215,216). The activated (bone marrow) macrophage may play a key role in suppression of erythroid growth. First, a negative correlation has been found between the levels of Hb and neopterin, a sensitive marker of macrophage activation (214). Second, activated bone marrow macrophages from patients with ACD inhibit growth of CFU-E *in vitro* (217,218). T-cells may also be involved as CFU-E growth is suppressed by peripheral T-cells from anaemic RA patients but not by T-cells from healthy controls (216). Activated macrophages and T-cells produce cytokines which may

inhibit erythroid growth (see below). Several reports show that erythoid proliferation *in vitro* is inhibited by serum from patients with inflammatory or malignant disorders (219,220). This might be explained by effects of circulating cytokines, directly or indirectly via stimulation of bone marrow macrophages. Therefore, inhibition of erythropoiesis in ACD might be mediated by cytokines, produced locally and/or systemically.

TNF is an important mediator of ACD. Serum TNF levels are elevated in a wide variety of disorders associated with ACD (see *1.2.2.1*). Moreover, TNF concentrations in the supernatant of bone marrow cultures from RA patients with ACD were significantly higher compared to nonanaemic patients (221). *In vitro*, TNF has inhibitory effects on human BFU-E and CFU-E proliferation (215,222). It was shown that TNF suppresses BFU-E growth in a dose-dependent manner in bone marrow from both RA patients with ACD and healthy subjects. This TNF-induced decrease in BFU-E count, which was more pronounced in ACD, could be counteracted by the addition of anti-TNF (215). *In vivo*, chronic TNF administration in the animal model induces a hypoproliferative, hypoferraemic anaemia, compatible with ACD in humans (223). A phase I trial with TNF in patients with cancer was accompanied by the development of anaemia, although not further classified (224).

IL-1 has also been implicated in ACD. In RA, serum IL-1 levels are significantly higher in anaemic patients compared to nonanaemics (225). IL-1 inhibits colony formation by BFU-E and CFU-E of normal human marrow *in vitro* (225). This might, however, be an indirect effect via induction of IFNγ (226). Chronic IL-1 administration to mice induces anaemia associated with suppression of CFU-E, presumably mediated through TNF (227).

IFNγ is thought to play a major role in inhibition of erythropoiesis in ACD. Increased serum neopterin levels in ACD reflect IFNγ-induced macrophage activation (214). *In vitro*, IFNγ has a direct suppressive effect on erythroid colony growth, in which it synergistically acts with TNF (226,228). In cancer patients, IFNγ treatment is accompanied by anaemia (229).

IL-6 may serve as a growth factor in erythropoiesis (41). IL-6 had inconsistent effects on erythroid growth in bone marrow of RA patients with and without ACD. However, addition of anti-IL-6 to these bone marrow cultures clearly inhibited erythroid colony growth (230). Thus, considering its possible stimulatory effects on erythropoiesis, IL-6 may have a counterregulatory role to other cytokines in ACD. Surprisingly, IL-6 administration in rats induced anaemia resulting from intestinal blood loss whereas no evidence was found for a suppressed erythropoiesis (231). Whether this mechanism is also operative in humans is unknown.

Other factors which may be involved in suppression of erythropoiesis in ACD include cytokines like IFNα and β and TGFβ (232) and APP such as ferritin and α1-antitrypsin (233,234). Their exact role, however, has yet to be established.

1.4.2.2 Iron metabolism

ACD is associated with profound changes in iron metabolism reflected by characteristic alterations in parameters of iron status (see **1.4.1**). Modulation of iron metabolism in ACD results in a decreased erythroblast iron availability which impairs erythropoiesis. First, this is mediated by iron redistribution to storage sites (Fig. 8), induced by the chronic inflammatory state as outlined in *1.3.5.2*. It must be stated that many studies on the effects of inflammation on iron metabolism involve animal models or *in vitro* studies. The number of ferrokinetic studies in humans is limited. As stated before, early MPS iron release is decreased in inflammation (193). In RA, ferrokinetic measurements showed conflicting results. Some studies demonstrate diminished iron reutilization (189,235,236), whereas others found normal MPS iron release in ACD (237,238). These differences might be explained by inappropriate patient selection. RA is often accompanied by iron deficiency and overlap between ACD and iron deficiency frequently occurs (213). It is clear that co-existing iron deficiency may influence studies on MPS iron kinetics in ACD. Nonetheless, iron retention by storage sites is believed to play a role in ACD.

Second, iron incorporation into erythroblasts may be decreased in ACD, concomitantly reducing iron availability. Incubation studies with ^{59}Fe-labelled ^{125}I-transferrin in bone marrow of anaemic and non-anaemic RA patients and healthy subjects have shown that both iron uptake and transferrin binding by erythroblasts are significantly decreased in ACD (239). This might rely on an impaired TfR expression by erythroblasts with respect to TfR number and/or affinity for its ligand. Alternatively, activated macrophages in ACD bone marrow cultures may have retained radiolabelled transferrin. This is, though, a less plausible explanation, considering differences in TfR number on macrophages and erythroid precursors. *In vivo*, TNF-induced anaemia in rats coincides with a decreased ^{59}Fe-incorporation into eythroid cells (223). In this thesis erythroid TfR expression is assessed in RA patients with and without ACD (see *1.5.2*).

In the inflammatory state changes may occur in transferrin glycosylation as described in *1.3.2.1*. Since this might influence functional properties of transferrin, it can be hypothesized that modulation of transferrin glycosylation may influence iron transport and donation to target tissues in inflammation. Whether changes in transferrin microheterogeneity in inflammatory disorders are related to disease activity and to the presence or absence of anaemia is presently unknown and is examined in this thesis (see *1.5.1*).

Finally, intestinal iron absorption may be impaired in inflammation and ACD. It was shown that mucosal iron uptake is significantly lower in RA patients with depleted iron stores as compared to subjects with an uncomplicated iron deficiency anaemia (240). The mechanism by which inflammation affects intestinal iron absorption has, however, not been elucidated yet.

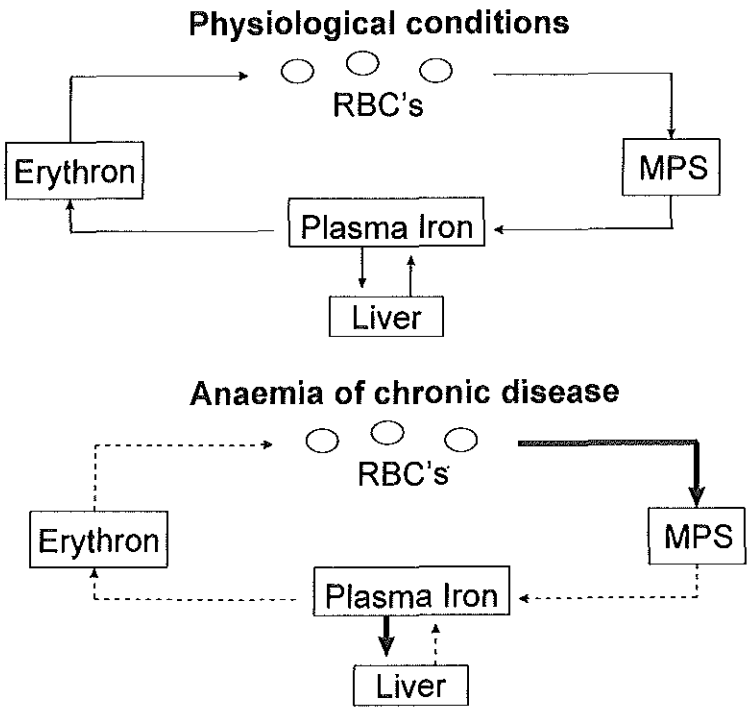


Figure 8. Iron recirculation in physiological conditions and ACD. In ACD iron is redistributed towards storage sites, presumably due to both an enhanced iron influx into MPS and liver and an impaired storage iron release. This results in hypoferraemia with a concomitant decreased erythroblast iron availability. RBC, red blood cell.

1.4.2.3 Erythropoietin

Erythropoietin [EPO], a glycoprotein predominantly synthesized by the kidney in response to hypoxia, is a growth factor involved in the regulation of erythropoiesis (241). Epo acts via a specific surface receptor on erythroid progenitor cells of both BFU-E and CFU-E and stimulates proliferation, differentiation, TfR expression and Hb synthesis (241,242). The mechanism by which EPO production is regulated is still unclear. It is postulated that EPO synthesis is modulated by a haeme protein which senses renal hypoxia (243). Oxygen is assumed to bind to ferrous iron of the haemeprotein and alters its conformation. EPO production would than depend on the conformational state of the haemeprotein.

Generally, serum EPO levels rise in response to anaemia if renal function is unaffected. In ACD, EPO responsiveness to the anaemia may be impaired as has been shown in ACD associated with chronic infectious, inflammatory and malignant disorders (244-247). In these conditions serum EPO levels increase, but are inappropriately low for the degree of anaemia. Although this relative EPO deficiency contributes to the pathogenesis of ACD, it is not a primary cause of ACD as EPO levels are still increased. In RA, serum EPO levels are equal in patients with and without ACD, which might imply that in nonanaemics an EPO rise prevents the development of anaemia (246). In patients with ACD, RA disease activity is usually higher compared to nonanaemics, suggesting that EPO responsiveness is modulated by the inflammatory response. *In vitro*, TNF, IL-1 and IFNγ inhibit EPO synthesis by human hepatoma cell lines in a dose-dependent manner (248-250). In addition, IL-1 inhibits EPO production in isolated hypoxic rat kidneys (251). Cytokines may thus affect EPO metabolism in ACD, although no data are available on cytokine effects on EPO production in humans.

The impaired EPO responsiveness in ACD may also be related to increased iron stores. In RA serum EPO levels correlate negatively with serum ferritin levels and in RA patients with ACD serum EPO levels are lower than in those with iron deficiency, despite comparable Hb levels (246). This might still be explained by a higher RA disease activity in ACD. However, iron chelation treatment of RA patients with ACD is followed by a rise in serum EPO and Hb levels associated with a decrease in serum ferritin levels, independent of disease activity (252). Thus, the blunted EPO response in ACD might be mediated by increased iron stores next to inhibitory effects of cytokines. It might be speculated that changes in body iron status modulate the EPO response to anaemia by influencing the regulatory function of the oxygen-sensing haemeprotein. This needs, however, further investigation.

Apart from a relative lack of endogenous EPO, responsiveness of erythroid progenitors to EPO may be decreased in ACD, although conflicting data exist on this subject. In some studies bone marrow sensitivity to EPO in ACD was reduced (253,254), whereas other studies found a normal response to EPO (255,256). There is, though, evidence that inhibition of erythropoiesis by cytokines may, at least in part, be mediated by interference with EPO responsiveness. *In vitro*, inhibition of human CFU-E growth by IFNγ could be counteracted by exposure to high EPO concentrations (257). Similarly, TNF-induced inhibition of CFU-E and BFU-E obtained from RA patients with ACD could be corrected with EPO (258). Indirect evidence for decreased bone marrow sensitivity to EPO in ACD is provided by the observation that treatment of ACD with recombinant human EPO [r-HuEPO] requires higher dosages to correct anaemia compared to treatment of the anaemia of chronic renal failure (see **1.4.4**).

1.4.2.4 Erythrocyte survival

Red blood cell [RBC] survival may be moderately decreased in ACD as has been shown in various inflammatory conditions (182,259). This might be attributed to an increased removal of RBCs by the MPS rather than to an intrinsic erythrocyte defect since normal RBCs transfused into patients had a shortened lifepan, whereas RBCs from patients transfused into healthy subjects had a normal survival time (182). In rats, TNF administration induces a decreased survival of RBCs (223). Increased clearance of nonsenescent RBCs in ACD may result from an increased phagocytic capacity of activated macrophages (260). In addition, RBC destruction is accelerated in the presence of fever (261).

1.4.3 Diagnosis

The diagnosis ACD can be made according to the following criteria: (a) presence of a chronic infectious, inflammatory or malignant disease; (b) low serum iron, transferrin and transferrin iron saturation values, elevated ferritin levels and relatively low reticulocyte count; (c) exclusion of other causes of anaemia (181). The main diagnostic problem is the differentiation of ACD from iron deficiency anaemia [IDA] and in particular the detection of co-existing iron deficiency [ID]. Examination of bone marrow iron content is regarded as the gold standard for assessment of body iron stores (139). Absence of stainable iron points to IDA, whereas normal to increased stainable iron is compatible with ACD. However, considering patient discomfort and costs it is desirable to assess body iron status by noninvasive measurements.

Apart from ferritin and transferrin, serological parameters are similarly affected in ACD and IDA (Table 9). Ferritin is a reliable marker to predict bone marrow iron stores in the absence of an acute phase response (139). Generally, serum ferritin levels below cut-off values of 15-25 µg/l are diagnostic of IDA. However, in inflammation ferritin behaves as an acute phase reactant and its levels are disproportionately increased and may thus mask concomitant ID. In ACD, ferritin levels usually exceed 100 µg/l when iron stores are adequate but may be within the normal range in the presence of co-existing ID (139).

An approach to predict IDA in inflammatory states is to use a higher cut-off point of serum ferritin, derived from simultaneous ferritin and stainable iron measurements in patients with ACD. Reported cut-off values below which iron stores are depleted range from 45 to 100 µg/l (262-266), which may in part be explained by the use of different ferritin test kits. However, ferritin levels above these cut-off points do not fully exclude co-existing ID (264,265). Diagnostic accuracy can be improved by combining parameters with their cut-off points as has been shown in RA for ferritin, MCV and transferrin (262). Nonetheless, ferritin levels between 20 and 100 µg/l remain a gray zone with a considerable overlap between ACD and IDA.

Table 9. Serological parameters in ACD and iron deficiency anaemia

Parameter	ACD	Iron deficiency anaemia
MCV	normal or decreased	decreased
RDW	normal or increased	increased
Serum iron	decreased	decreased
Serum transferrin	decreased	increased
Transferrin iron %	decreased	decreased
Serum ferritin	increased	decreased
Serum lactoferrin	normal or increased	normal
Serum TfR	normal (?)	increased
Erythrocyte ferritin	normal or decreased	decreased
Erythrocyte zinc protoporphyrin	normal or increased	increased

Abbreviations: ACD, anaemia of chronic disease; MCV, mean corpuscular volume; RDW, red cell distribution width; TfR, transferrin receptor (183).

As outlined in **1.3.4** the sTfR is a reliable index of tissue iron stores (179). The sTfR assay is an adequate method to differentiate uncomplicated IDA from ACD (180). Compared to serum ferritin, sTfR levels may have the advantage of being less sensitive to the inflammatory response. It might be speculated that the sTfR is a useful tool to assess body iron stores in inflammatory states, which is examined in this thesis (see *1.5.3*).

1.4.4 Treatment

Since ACD is usually a mild and nonprogressive anaemia, treatment is rarely necessary. Indications for treatment are the presence of co-existing angina pectoris, heart failure and respiratory insufficiency as well as major surgery. Relative indications include pregnancy, a decreased exercise tolerance, fatique, palpitations and vertigo (213). According to the relation between inflammatory activity and the development of ACD, the most rational approach to

correct the anaemia is to treat the underlying disease. Indeed, in RA for instance, a reduced disease activity following antirheumatic treatment is parallelled by a considerable rise in Hb levels (267).

R-HuEPO is an effective treatment for the anaemia of chronic renal failure, concomitantly improving exercise capacity and quality of life (268). Based on the concept of an impaired EPO response and a decreased marrow sensitivity to EPO, trials have been carried out with r-HuEPO in patients with ACD. Treatment with r-HuEPO of ACD associated with chronic disorders like RA, AIDS and cancer resulted in improvement or correction of anaemia (269-272). Interestingly, in patients with RA r-HuEPO had also beneficial effects on RA disease activity, pointing to a possible immuno-modulatory role for EPO (270).

Another approach to the management of ACD is treatment with iron chelators, since these agents can mobilize iron stored in MPS and liver. Parenteral administration of desferrioxamine in patients with RA was followed by an increase in Hb (273). In a preliminary study in RA, treatment with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one [L1] resulted in a Hb rise as well (252). Possible explanations for an improved erythropoiesis after iron chelation treatment are an increased erythroblast iron availability, an increased EPO response and a decreased disease activity. Larger and long-term trials are needed to establish a role for iron chelation in the treatment of ACD.

1.5 Summary and aims of the thesis

The acute phase response involves a pattern of physiological reactions in response to various stimuli. Regulation of this systemic response is thought to be mediated by the activated cytokine network. Within this network TNF and IL-1 act as proximal mediators which induce a cascade of other cytokines, e.g. IL-6. Several mechanisms exist to counteract the inflammatory response, e.g. the release of soluble cytokine receptors. One of the major characteristics of the acute phase response is the change in plasma protein synthesis and glycosylation. Cytokines modulate APP synthesis and post-translational modification *in vitro*, their *in vivo* effects on APP in humans, however, are largely unknown. In the inflammatory state, complex interactions exist between immune and endocrine responses. For instance, proinflammatory cytokines activate the HPA-axis. Glucocorticoids, in turn, down-regulate cytokine production. Another major endocrine adaption in the acute and chronic inflammatory state involves modulation of the thyroid hormone axis resulting in the so-called euthyroid sick syndrome. Cytokines may have an important role in the induction of this euthyroid sick syndrome, which may serve as a

mechanism to counteract excessive catabolism in inflammatory responses.

Conversion of the acute phase response to the chronic inflammatory state coincides with the development of ACD. Although the pathogenesis of ACD is still unclear, several sequelae of the acute phase response such as cytokine release and alterations in iron metabolism are likely to be involved. Erythropoiesis is impaired in ACD, based on inhibition of erythroid growth and a decreased iron availability, which is amplified by an impaired EPO response to the anaemia. Cytokines such as TNF, IL-1 and IFNγ may play a key role in these pathophysiological mechanisms, although many of their postulated effects have yet to be established.

One of the major pathogenetic factors in ACD is a decreased erythroblast iron availability. First, this is based on redistribution of iron to liver and MPS resulting in hypoferraemia associated with elevated ferritin levels. Iron retention by storage sites may in part be mediated by a primary induction of apoferritin with a concomitant entrapment of labile iron. TNF, IL-1, IL-6 and IFNγ induce hypoferraemia in experimental animals and modulate iron handling by various cell types *in vitro*. The effects of cytokines on iron metabolism in humans, however, are presently unknown. Second, transferrin binding and iron uptake by erythroid precursors may be decreased in ACD. It is not known, however, whether this is related to an impaired TfR expression by erythroblasts. Transferrin acts as a negative APP and in the inflammatory state changes may occur in transferrin glycosylation, possibly affecting its functional properties. It might be speculated that transferrin glycosylation is modulated in ACD, which may in turn influence iron metabolism with respect to e.g. iron transport.

The diagnosis ACD is made according to its characteristic iron status and by exclusion of other causes of anaemia. It can be difficult, however, to exclude co-existing iron deficiency in inflammatory conditions accompanied by ACD since serum ferritin acts as an APP. Serum TfR concentrations are a reliable index of tissue iron stores and erythroid proliferation and may be a promising tool in the detection of iron deficiency in inflammation. ACD usually resolves after recovery of the underlying disorder and treatment is rarely indicated. ACD can be corrected by treatment with r-HuEPO and possibly by administration of iron chelators.

The aim of the present thesis is to obtain more insight in the regulation of the acute phase response and the pathogenesis of ACD with respect to the role of cytokines, endocrine systems and iron metabolism. In part I, patients with RA are studied as model for the chronic inflammatory state. In RA patients with and without ACD the following aspects are examined: (a) iron status and transferrin glycosylation in relation to disease activity and cytokine levels; (b) transferrin receptor expression by erythroblasts; (c) sTfR levels and the effects of treatment with r-HuEPO and iron chelation treatment. In part II TNF administration in cancer patients is used as a concept to examine the acute inflammatory state. Patients with locally advanced

extremity sarcomas and melanomas can successfully be treated by isolated limb perfusion [ILP] with high-dose recombinant human TNF [r-HuTNF] and melphalan (274). After this procedure, resorption of residual TNF from the perfused limb results in high systemic TNF levels, providing a model to examine *in vivo* effects of TNF. In these patients treated with ILP the effects of TNF-infusion on the following items are examined: (a) induction of cytokines and the acute phase response; (b) aspects of iron metabolism (related to part I) in the acute phase response; (c) cortisol and thyroid hormone metabolism in the acute phase response.

Aims of the thesis

Part I: Iron metabolism in the anaemia of chronic disease: rheumatoid arthritis as model for the chronic inflammatory state

1.5.1 To examine iron status and transferrin microheterogeneity in RA patients with ACD in relation to disease activity and cytokine levels as an altered transferrin glycosylation might play a pathophysiological role in ACD.

1.5.2 To investigate whether an impaired transferrin receptor expression by erythroblasts is involved in the pathogenesis of ACD in RA.

1.5.3 To examine a possible diagnostic role for the serum transferrin receptor [sTfR] in the detection of iron deficiency in RA.

1.5.4 To study the effects of erythropoietin and iron chelation treatment of RA patients with ACD on sTfR levels in relation to changes in erythropoiesis and iron status.

Part II: Iron metabolism and endocrine systems in the acute phase response: TNF administration to cancer patients as model for the acute inflammatory state

1.5.5 To examine the *in vivo* effects of TNF on the acute phase response with respect to induction of IL-6, soluble TNF receptors and acute phase protein production.

1.5.6 To investigate the mechanism of hypoferraemia in the acute phase response by assessing kinetics of iron and iron binding proteins.

1.5.7 To study the effects of TNF on iron, iron binding proteins, transferrin glycosylation and sTfR concentrations in the acute phase response.

1.5.8 To examine the effects of TNF on thyroid hormone and cortisol metabolism in the acute phase response.

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Chapter 2

PATIENTS AND METHODS

2.1 Patients

In chapter 3, 4 and 5 patients were studied with RA, fitting the revised criteria of the American Rheumatism Association (1). All patients gave written informed consent before the study. The diagnosis ACD was made based on normal to increased bone marrow iron content (see 2.8.1) and the exclusion of other causes of anaemia by: (a) history-taking: present or past ulcer disease, other gastrointestinal disease or complaints, hypermenorrhoea and haematuria; (b) normal values of serum vitamin B12 and folic acid concentrations, normal Coombs test and creatinine clearance and absence of microscopic haematuria or occult blood loss in stools. Patients were considered as iron deficient if stainable bone marrow iron was absent.

In chapter 6, 7 and 8 patients were studied with irresectable soft tissue sarcoma and with melanoma with multiple in transit metastases. Written informed consent was obtained from all patients.

Demographic patient characteristics are described separately in each chapter. All study protocols were approved by the Medical Ethical Committee of the Dr. Daniel den Hoed Cancer Center.

2.2 Parameters of iron status

Reference values are given in parentheses. In chapter 3, 4 and 5 serum iron was determined by a colorimetric analysis of a ferrous iron/ferene complex at 595 nm (Instruchemie, Hilversum, The Netherlands; 14-30 µmol/l). In chapter 7 serum iron was measured by the ferrozine method as developed for Boehringer Mannheim/Hitachi systems (10-32 µmol/l).

In chapter 3, 4 and 5 serum transferrin was measured by a nephelometric assay using a Baker nephelometer 420 (Ablon Medical Systems, Leusden, The Netherlands, 44-80 µmol/l) and in chapter 6 and 7 by an in-house nephelometric assay (Department of Auto-immune Diseases, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands; 2-4 g/l). Transferrin iron saturation was determined by means of the ratio, serum iron/total iron binding capacity.

Serum ferritin was determined with a solid phase enzyme immunoassay (Ferrizyme, Abott Labs, Chicago, USA) in chapter 3, 4 and 5 (20-150 µg/l) and using an enzyme linked immunosorbent assay [ELISA] in chapter 6 and 7 (50-110 µmol/l).

2.3 Erythrocyte parameters

Haemoglobin was measured by a modified cyanmethemoglobin colorimetric analysis at 540 nm. (7.4-10.9 mmol/l).

Mean corpuscular volume [MCV] was determined by calculating the ratio of haematocrite

and number of erythrocytes (80-96 fl).

Reticulocyte count was measured by vital staining with brilliant cresyl blue and expressed as a ratio of red cells (0/00).

2.4 Parameters of disease activity and acute phase proteins

Erythrocyte sedimentation rate [ESR] was determined by means of the Westergren method (<10 mm/h).

C1q binding was measured according to the method described by Zubler and Lambert (2) (<7 %).

C-reactive protein [CRP] was determined by radial immunodiffusion (Behring Werke, Marburg, Germany; <6 mg/l).

α1-Antitrypsin (1.7-3.2 g/l), α1-acid-glycoprotein (0.4-0.9 g/l), albumin (35-55 g/l) and complement factors C3 (0.9-1.8 g/l) and C4 (150-400 mg/l) were measured by in-house nephelometric assays (Department of Auto-immune Diseases, CLB, Amsterdam, The Netherlands).

2.5 Cytokines and cytokine receptors

2.5.1 TNF

For determining the serum levels of TNF, serum was directly centrifugated and frozen. In chapter 3, serum TNF concentrations were measured by means of an immunoradiometrical assay using monoclonal antibodies against distinct epitopes of TNF (IRE-Medgenix, Fleurus, Belgium; < 8pg/ml). In chapter 6-8, Serum TNF levels were determined by a TNF specific sandwich ELISA (CLB, Amsterdam, The Netherlands) as decribed previously (3). In this assay a monoclonal antibody against human TNF is used as a coating antibody with a biotinylated anti-TNF monoclonal antibody as a second step. Streptavidin-polymerized horseradish peroxidase is used as a conjugate. Reference values, which result from TNF measurements in 100 healthy individuals, are < 50 pg/ml (mean +2SD).

2.5.2 IL-6

In chapter 3, IL-6 levels were measured using the B9 assay as described before (4). In short, samples are titrated by means of serial dilutions in culture medium. Samples then are added to $5 \cdot 10^3$ B9 cells and compared to a standard IL-6 preparation. Proliferation is determined after 3 days by ^{3}H-thymidine incorporation. IL-6 concentrations are expressed in U/ml and 1 U/ml reflects the concentration that induces half maximal proliferation. One unit equals about 1

pg/ml of IL-6. Normal IL-6 values are < 10 pg/ml. In chapter 6-8, IL-6 concentrations were determined by an ELISA as described in (5). In short, serial dilutions of serum samples are added to microtiter plates, which are coated with a purified monoclonal antibody against IL-6. Bound IL-6 is detected by biotinylated affinity purified polyclonal sheep anti-IL-6. Normal values are < 10 pg/ml.

2.5.3 Soluble TNF receptors

Soluble TNF receptors [sTNF-R] 55 and 75 were measured by sandwich ELISA's as described before (6). In short, serum is added to immunoassay plates which are coated with MR1-1 (anti-TNF-R55) or with MR2-2 (Anti-TNF-R75). Subsequently, the samples are incubated with specific biotin-labeled rabbit anti-sTNF-R antiserum. Then, after washing, peroxidase labelled streptavidin is added and peroxidase acitivity is determined by the addition of 3,3',5,5'-tetramethylbenzidine substrate. sTNF-Rs are quantitated by photospectrometry (450 nm). The lower detection limit of this ELISA is 100-200 pg/ml. Normal values are < 2 ng/ml.

2.6 Hormones

2.6.1 Thyroid hormones, TSH and TBG

Plasma T4 (70-150 nmol/l), T3 (1.3-2.7 nmol/l) and rT3 (0.11-0.44 nmol/l) were measured with in-house radio-immuno assays [RIA] (Laboratory for Endocrinology, Academical Medical Center, Amsterdam, The Netherlands) (7).

FT4 was measured using SPAC FT4-fraction (Byk-Sangtec Diagnostica, Dietzenbach, Germany; 10.0-23.0 pmol/l). T3 uptake was determined by a MMA kit (Kodak Clinical Diagnostics, Amersham, England; 0.84-1.11).

TSH was measured by a third generation assay IMx (Abbott, Laboratories, North Chicago, IL; 0.4-4.0 mU/l).

TBG was measured using a RIA (Eiken Chemical CO, Tokyo, Japan; 200-650 nmol/l).

2.6.2 Cortisol

Cortisol was measured by a fluorescence polarization immunoassay with Technical Device X (Abbott Laboratories, Chicago, IL; 0.22-0.65 µmol/l).

2.7 Transferrin microheterogeneity

Transferrin microheterogeneity in chapter 3 is assessed by crossed immunoelectrophoresis [CIE] as described previously (8). Before CIE, 50 µl serum is incubated with 50 µl of 25 mM

$NaHCO_3$ and 2 µl of 10 mM Fe(III)citrate to ensure complete iron saturation of serum transferrin. In the first phase, transferrin subfractions are separated by isoelectric focusing on polyacrylamide gelstrips carrying an immobilized pH-gradient of 4.9-6.1 (LKB, Bromma, Sweden). Anode and cathode strips, soaked in 10 mM glutamic acid and 10 mM NaOH respectively, are positioned at the ends of the gel strips. Subsequently, 2 µl serum is pipetted at the cathodal side of the strips, followed by application of 1000 V during 18 h using a LKB Multiphor apparatus and a LKB 2103 power supply. The second phase, which is run perpendicularly to the first, involves a rocket immunoelectrophoresis. A preheated 1 % w/v solution of Agarose-M (LKB) in 24 mM sodium barbiturate-HCl buffer (pH 8.7) is mixed with rabbit anti-human transferrin antibody (Dako, Copenhagen, Denmark) at 55 °C. This mixture is spread on a preheated glass plate followed by positioning of the gelstrips in the agarose layer. Subsequently, CIE is carried out at a voltage of 12-13 V/cm during 4 h at 10 °C. The gels are mounted on GelBond film, air-dried and then stained in a solution of 0.5 % Amido Black.

With CIE nine transferrin fractions can be separated based on differences in sialic acid content. These transferrin isotypes are termed 0 to 8 sialo-transferrin and the relative proportions are quantitated by measurement of the areas enclosed by the immunoelectrophoretic peaks. Relative concentrations are derived from combining these data with total transferrin concentration. All microheterogeneity patterns are assessed in duplicate.

In chapter 7 transferrin microheterogeneity is assessed by isoelectric focusing [IEF] using the PhastSystem (Pharmacia, Sweden), which was shown to be an efficient, but less labour intensive, method to examine transferrin microheterogeneity (9). A part of an immobiline DryPlate pH 4-7 (LKB, Bromma, Sweden) is cut out to obtain a gel with pH gradient 5-6 and with the same size as a PhastGel. This gel is rehydrated by application of 1 ml 20 % (v/v) glycerol on the gel surface. At the cathode and anode side of the gel strips are applied saturated with 2 % (w/v) Ampholine pH 6-8 and 2 % (w/v) Ampholine pH 2.5-4.5 respectively. Serum samples, after iron saturation, are applied to the gel by a sample applicator. The following program was used for IEF:

Separation step x.1	2000 V	2.0 mA	2.0 W	15 °C	485 Vh
Separation step x.2	200 V	0.2 mA	0.2 W	15 °C	15 Vh
Separation step x.3	2000 V	2.0 mA	2.0 W	15 °C	1300 Vh

Sample applicator: - down at: x.2, 0 Vh
- up at: x.3, 0 Vh

Directly after IEF, the gel surface is incubated with 100 µl rabbit anti-human transferrin antibody (Dako, Copenhagen, Denmark) during 20 min. at room temperature. Then, the gel is washed overnight in 0.15 M NaCl with repeated replacement of washing solution. After incubation during 20 min. with a 12 % trichloroacetic acid solution the immunoprecipitated bands are stained with Coomassie Brilliant Blue G-250. Sialo-transferrin bands are quantitated with an Ultroscan Xlaser Densitometer connected with GelScan XL (2.1) software (Pharmacia Sweden). Analogous to CIE, the microheterogeneity pattern as assessed by IEF can identify 0 to 8 sialo-transferrin subfractions. All microheterogeneity patterns are determined in duplicate.

2.8 Bone marrow studies

2.8.1 Bone marrow iron content

Bone marow was aspirated by means of sternal or posterior iliac crest puncture. Bone marrow iron content was assessed after staining with Perl's Prussian blue using a semi-quantitative scale: 0 = no stainable iron; 0-1 = minimal amount of iron; 2 = slight small and patchy iron content; 3-4 = normal to increased stainable iron. Iron deficiency is defined by a stainable iron content of 0-1 (10).

2.8.2 Bone marrow preparation

A cell suspension was prepared from 20 ml bone marrow collected in Hank's balanced salt solution [HBSS] with heparin diluted in HBSS and layered over a Ficoll gradient (1.077 g/cm^2, Nycomed, Oslo, Norway), in order to remove polymorphonuclear cells. After centrifugation at 1,200 rpm and 4 °C during 7 minutes, the cells were washed twice in Dulbecco's Eagle Modified Medium (DMEM) and resuspended in DMEM (11). Total cell count was assessed using a Burker counting chamber. A differential count was carried out using a cytospin and May-Grunwald staining. Trypan blue staining (Sigma) was used in order to assess cell viability.

2.8.3 Labeling of transferrin

Human transferrin (Behring Werke, Marburg, Germany) was saturated with iron by means of the iron-nitrilotriaceticacid [Fe-NTA] method (12), using a sodium bicarbonate solution (0.05 M) as an anion donor which is necessary for Fe^{3+} binding to transferrin (13). Unbound Fe-NTA was removed by G25 chromatography with phosphate buffered saline [PBS] followed by extensive dialysis against PBS. Diferric transferrin was subsequently labeled with ^{125}I (Amersham Radiochemical Centre, UK) using Iodogen reagent (Pierce Chemicals, Rockford, IL) as catalyst. The surplus of ^{125}I was separated by gelfiltration using a PD-10 column (Pharmacia)

which was equilibrated with a 0.1 M sodium bicarbonate solution. Radioactive peakfractions were pooled followed by extensive dialysis against PBS, pH 8,2. Iron saturation and protein concentration were measured by the absorbance at 470 nm and 280 nm respectively. The ratio 470/280 was never < 0.045. The mean specific activity of ^{125}I-transferrin was 2.5 .10^6 cpm/nmol.

2.8.4 Binding assays

^{125}I-labeled transferrin was incubated with 1.0 to 5.9 .10^6 bone marrow mononuclear cells suspended in an incubation mixture of DMEM pH 7.4 containing 5% Fetal Calf Serum and DNA-se (30 mg/100 ml). The concentration of transferrin ranged from 0.02 nmol/ml to 2 nmol/ml. The final incubation volume was 1 ml. Incubation was carried out at 0 °C for 60 min. The cells were then centrifugated at 1200 rpm and 4 °C during 7 minutes after which the supernatant was removed. Subsequently, the cells were washed three times in cold PBS and radioactivity of the cell bound ligand was measured using a Packard-autogamma 500.C. (13). Non-specific binding, usually less than 10 %, was determined by measuring the radioactivity bound to the cells in the presence of a 100-fold excess of unlabeled transferrin and all specific binding data were corrected accordingly. All measurements were carried out in duplicate. The association constant (Ka) and the number of transferrin receptors per erythroblast were calculated by the method of Scatchard (14).

2.9 Serum transferrin receptor assay

In chapter 5, sTfR concentrations were determined using a modified ELISA as described before (15). Two non-cross-reactive monoclonal antibodies, OKT 9 and J 64, are used (16). The wells of ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) are incubated overnight with OKT 9 at a pH of 9.6 after which free binding sites are blocked with 100 µl 0.5 % bovine serum albumin for 30 min. Subsequently, 100 µl of various serum sample dilutions is pipetted into the coated wells and incubated for 60-90 min. Then 100 µl (2 µg/ml) biotinylated J 64 in PBS Tween and, after washing, 100 µl of a mixture of streptavidin and biotinylated horseradish peroxidase (1:2000 in PBS Tween) (Amersham, Germany) are added. Thirty minutes later, after three washing procedures, 100 µl (1 mg/ml) orthophenylendiamine (OPD) in OPD buffer mixed with 0.25 µl 30 % H_2O_2/mg OPD is added. As soon as color intensity is sufficient (generally after 10-15 min.), the reaction is blocked by the addition of 100 µl 1 M HCl. A spectrophotometer is used (492 nm.) for quantitative measurement of sTfR. The samples are compared with a standard preparation in PBS Tween (17). In 20 healthy blood donors median sTfR was 2.8 (1.7-3.5) mg/l.

In chapter 7 sTfR concentratons were also measured with an ELISA based on the double antibody sandwich method (RAMCO assay, DPC, Apeldoorn, The Netherlands). The test principle is similar to the above described ELISA. Samples of 50 µl of serum (prediluted 1:100) and standards are pipetted in micowells which are precoated with a polyclonal antibody to the TfR, followed by incubation for 2 h at room temperature with a murine monoclonal anti-TfR antibody which is conjugated to horseradish peroxidase. After four washing steps, chromogen TMB is added as a substrate and the subsequent colour reaction is terminated after 30 min. by addition of 50 µl acid stop solution. Absorbance of individual wells with serum and standards is measured at 450 nm. A standard curve is made by plotting the absorbance versus the concentration of the standards. The sTfR concentration in a serum sample can be determined in the standard curve by comparison of the sample's absorbance value. Analysis of sera obtained from 62 healthy individuals revealed normal values of 4.94 ± 1.64 mg/l (18).

2.10 Isolated limb perfusion

The method of regional isolated limb perfusion [ILP] for treatment of melanoma or sarcoma has previously been described in detail (19-21). Patients are pretreated daily with 0.2 mg recombinant human IFN γ (r-HuIFNγ, Boehringer Ingelheim, Germany) subcutaneously on the two days before ILP. The surgical procedure is carried out under general anesthesia using intravenous administration of propofol (Zeneca, Caponago, Italy), pancuronium bromide (Organon Technica, Oss, The Netherlands) and sufentanil (Janssen Pharmaceutica, Beerse, Belgium) and inhalation of isoflurane (Abbott, Queensborough, England), nitrous oxyde and oxygen. The extra-corporeal circuit is primed with erythrocyte concentrate and fresh frozen plasma diluted with Hartman electrolyte solution and polygeline solution (Haemaccel, Hoechst AG, Mannheim, Germany). Heparin (Novo-Nordisk AS, Rud, Norway) was used as anticoagulant, both in the priming solution (3 IU/ml) and systemically (200 IU/kg body weight). After cannulation of the local vasculature, perfusion takes place via an extra-corporeal circuit (Fig. 1) at mild hyperthermia (40°C). The limb is perfused during 90 minutes with recombinant human TNF (r-HuTNF, Boehringer, Ingelheim, Germany; 2 mg/arm or 4mg/leg) and 0.2 mg r-HuIFNγ. Melphalan (Wellcome, Beckenham, England; 13 mg L^{-1} arm volume or 10 mg L^{-1} leg volume) is administrated after 30 minutes. In the study described in chapter 8 ILP is carried out without r-HuIFNγ and without pretreatment with r-HuIFNγ as it was shown that r-HuIFNγ, although it may theoretically potentiate the effect of rTNF, did not to have additional value in anti-tumor treatment (20,21).

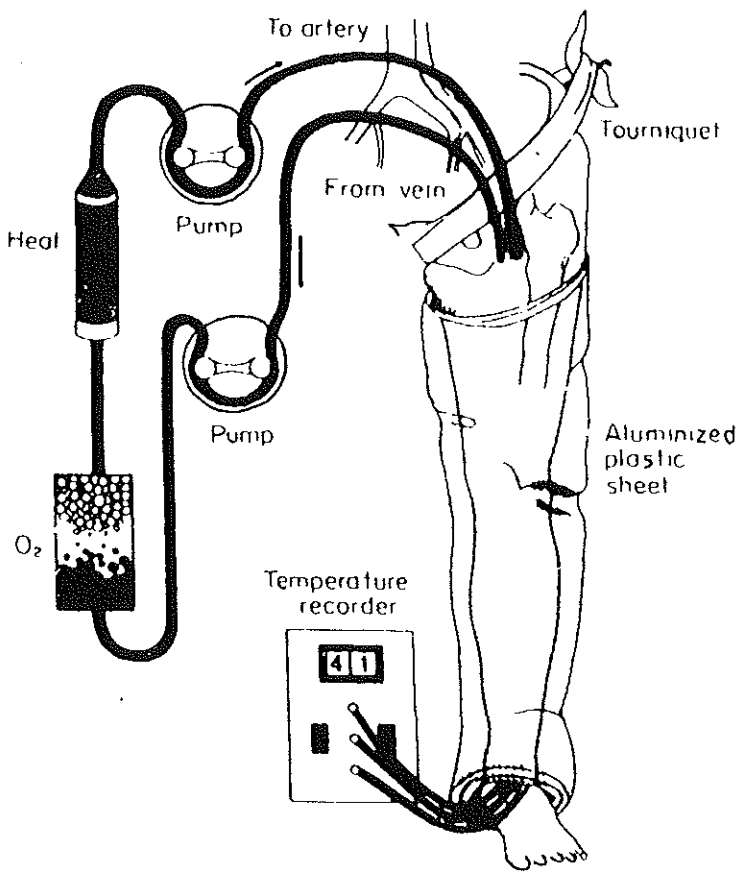

Figure 1. Schematic representation of the isolated limb perfusion procedure as performed by Eggermont et al. (ref. 20,21).

ILP is followed by drainage of the perfusate from the limb and a washout procedure with 2-4 l of 6% dextran 70 solution (Macrodex, NBPI BV, Amstelveen, The Netherlands). Subsequently, the limb is reconnected to the systemic circulation and 1 mg/kg protamin sulphate (Novo-Nordisk AS, Rud, Norway) is administrated to antagonize heparin. Perioperatively, all patients receive 2 units of erythrocyte concentrate, to replace blood loss due to the washout procedure

of the limb, and 3 l of isotonic saline intravenously. After the ILP procedure patients are extubated and observed at the intensive care unit for 24 hours. Postoperatively, prophylactic anticoagulation with acenocumarol (Ciba-Geigy AG, Basel, Switzerland) is started as well as subcutaneous administration of 5,000 units calparin (Sanofi Winthrop, Nôtredame de Bondeville, France) twice daily until adequate anticoagulation is achieved.

Blood was obtained from a peripheral vessel, which was not located in the perfused limb nor used for fluid administration. Blood samples were taken during pretreatment, at baseline and sequentially after the end of ILP which is defined as the moment of reconnection of the limb to the systemic circulation by releasing the tourniquet (see for specific time points chapter 6, 7 and 8).

2.11 Statistics

In chapter 3-5 the Student-t-test was used for comparison of normally distributed parameters, whereas non-parametric data were analyzed by the Mann Withney U test. Correlation of data was carried out by Spearman's rank correlation test. In chapter 6-8 serial measurements of various parameters were tested using one way analysis of variance [ANOVA] for repeated measures or Friedman's repeated measures ANOVA on ranks when appropriate. In this design, subjects serve as their own controls. After an ANOVA has resulted in a significant F test, post hoc comparisons are made with Dunnett's test for multiple comparisons. In this test, post-treatment data are compared with the data at baseline (22). $P < 0.05$ was considered to represent statistical significance.

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Part I

IRON METABOLISM IN THE ANAEMIA OF CHRONIC DISEASE

Rheumatoid arthritis as model for the chronic inflammatory state

Chapter 3

TRANSFERRIN MICROHETEROGENEITY IN RHEUMATOID ARTHRITIS

Relation with anaemia of chronic disease, disease activity and cytokines

Introduction

Chronic infectious, inflammatory and malignant disorders are often accompanied by anaemia defined as anaemia of chronic disease [ACD] (1-3). The pathogenesis of ACD is complex and only partly understood. ACD is frequently associated with active rheumatoid arthritis [RA] (2) and many studies on the pathogenesis of ACD have been carried out in patients with RA. Postulated concepts include: (a) inhibition of erythropoiesis by proinflammatory cytokines like TNF, IL-1 and IFNγ (4-7); (b) an impaired erythropoietin [EPO] response to the anaemia (8,9); (c) modulation of iron metabolism resulting in a decreased erythroblast iron availability. First, this is based on redistribution of iron towards storage sites, possibly mediated by cytokines, reflected in hypoferraemia and increased ferritin levels (10-12). Second, iron uptake by erythroblasts may be decreased (13).

Iron fluxes between different compartments are thought to rely predominantly on transport by transferrin (14). Transferrin iron saturation, the affinity of transferrin for its receptor and the number of transferrin receptors on erythroblasts determine erythroblast iron uptake in this scheme. The microheterogeneity of transferrin refers to structural variation in its carbohydrate moiety which results in a limited number of transferrin isotypes that can be distinguished and quantitated electrophoretically (15). Alterations in the relative proportions of the transferrin variants, reflecting changes in glycosylation, have been described in pregnancy and in several chronic diseases including RA, haemochromatosis and cancer (15,16). Since changes in glycosylation may influence functional properties of transferrin (14,17,18), modulation of transferrin microheterogeneity may influence iron delivery to target organs.

In acute and chronic inflammatory conditions, increased synthesis of several positive acute phase proteins [APP] has been shown to be accompanied by changes in their microheterogeneity (19-24). *In vitro,* both synthesis and glycosylation of APP are modulated by cytokines such as TNF, IL1, and IL-6 (25-29). In RA, the existence of both ACD (9) and alterations in glycosylation patterns of several APP appear to correlate with disease activity (20,21). Transferrin is considered to be a negative APP (25), indeed, in patients with active RA and ACD transferrin levels are frequently decreased (2). It is not known, however, whether this is associated with changes in transferrin microheterogeneity nor whether cytokines influence transferrin glycosylation in the acute phase response.

The aim of this study is to assess iron status and transferrin microheterogeneity in patients with RA in relation to ACD, disease activity and cytokine levels. First, since changes in transferrin glycosylation, which have been postulated to alter iron transport, may be present in ACD. Second, since TNF and IL-6, which have been implicated as mediators of the acute

phase response and ACD, may modulate iron metabolism in ACD with respect to induction of hypoferraemia and regulation of transferrin synthesis and glycosylation.

Patients and Methods

Patients

Serum was obtained from 21 healthy volunteers (controls) and from 25 patients with RA fitting the revised ARA-criteria (30), after acquiring written informed consent. These patients were divided into three groups: group 1: 9 nonanaemic patients (age: 54±5 years, disease duration: 6±3 years); group 2: 7 patients with iron deficiency anaemia based on the absence of stainable bone marrow iron (age: 56±5 years, disease duration 6±3 years); group 3: 9 patients with ACD based on normal to increased stainable bone marrow iron and the exclusion of other causes of anaemia (see 2.1) (age: 59±7 years, disease duration: 8±4 years). Mean age, sex and disease duration did not differ significantly among the three RA groups. All patients used non-steroidal antiinflammatory drugs, whereas 18 (72%) were on long-acting antirheumatic drugs. Clinical disease activity was highest in group 3 and lowest in group 1.

Laboratory procedures

Erythrocyte parameters and iron status

Determined were: Hb, Ht, reticulocytes, MCV, serum iron, transferrin, ferritin and stainable bone marrow iron (see 2.2, 2.3, 2.8).

Disease activity and cytokines

Serological disease activity was assessed by ESR, CRP and C1qba (see 2.4). TNF levels were determined immunoradiometrically and IL-6 levels were measured in the B9 assay (see 2.5).

Assessment of transferrin microheterogeneity

Transferrin microheterogeneity was assessed by crossed immunoelectrophoresis [CIE]. This technique is described in detail in 2.7. In short: in the first phase transferrin subfractions are separated by isoelectric focusing on polyacrylamide gelstrips carrying an immobilized pH-gradient. The second phase involves a rocket immunoelectrophoresis of all transferrin fractions simultaneously. This results in a pattern in which 9 transferrin fractions are separated due to differences in sialic acid content. An increased sialylation, i.e. an increase in transferrin frac-

tions with 5 to 8 sialic acid residues, indicates an increased branching of the glycans attached to the protein (15). The relative proportions were ascertained by measurement of the areas enclosed by the immunoelectrophoretic peaks, and relative concentrations were derived from combining these data with total transferrin concentration.

Data obtained were divided into three subgroups: the low sialylated transferrin fractions (LSTf, the sum of 0-, 1-, 2- and 3-sialotransferrin), the 4-sialotransferrin fraction (4-STf, as this is the predominant fraction in normal serum) and the highly sialylated transferrin fractions (HSTf, the sum of 5-to 8-sialotransferrin). This simplifying classification was based on earlier observations that within these 3 groups, changes occuring in the relative amounts of transferrin subfractions display similar trends (15).

Results

Iron status, RA-disease activity and cytokine levels

Patient characteristics are shown in Table 1. Compared to group 1 (nonanaemics) serum transferrin levels are significantly lower in group 3 (ACD) and significantly elevated in group 2 (iron deficiency anaemia). In all groups, however, transferrin levels are lower than in controls (mean 38.2±2.4 μmol/l). Transferrin concentrations correlate with TNF ($r=-0.55$, $p<0.025$) and IL-6 ($r=-0.68$, $p<0.025$) concentrations. Serum iron levels are decreased in all groups ($p<0.05$ compared to controls), but lowest in group 3 (NS) and correlate with TNF values ($r=-0.52$, $p<0.05$). Ferritin levels are elevated in group 3 ($p<0.01$) and decreased in group 2 ($p<0.05$) compared to group 1. A correlation was found between ferritin and IL-6 ($r=0.75$, $p<0.005$). The values of the iron status in the control group are within the normal range.

Disease activity, as assessed by ESR (although it is known that ESR and Ht correlate negatively to some extent), CRP and C1qba is highest in group 3, intermediate in group 2 and lowest in group 1. Serum TNF and IL-6 concentrations are significantly elevated in group 3 compared to group 1. Both TNF ($r=0.64$, $p<0.005$) and IL-6 ($r=0.55$, $p<0.05$) levels correlate with ESR values. IL-6 concentrations correlate with CRP levels ($r=0.53$, $p<0.05$).

Table 1a. Patient characteristics.

	1. Nonanaemics	2. Iron deficiency	3. ACD
Erythocyte parameters			
Hb (7.4-10.9 mmol/l)	8.0 (7.7-8.7)	6.5 (4.3-7.1)	6.6 (5.7-6.9)
Ht (0.36-0.51 l/l)	0.39 (0.35-0.42)	0.34 (0.24-0.37)	0.31 (0.28-0.35)
MCV (80-96 fl)	88 (80-116)	85 (61-95)	87 (80-98)
Reticulocytes (0/00)	13 (1-39)	11 (5-26)	17 (1-30)
Iron status			
Iron (14-30 µmol/l)	6 (1-11)	6 (2-10)	4 (2-17)
Transferrin (25-55 µmol/l)	28 (20-39)	32* (26-41)	23† (18-32)
Ferritin (20-150 µg/l)	50 (10-326)	10† (10-53)	125‡ (89-432)

*Erythrocyte variables and iron status in patients with rheumatoid arthritis without anaemia (group 1), with iron deficiency anaemia (group 2), and with anaemia of chronic disease (ACD, group 3). Data are expressed as median with range. *$p<0.10$, $^{\dagger}p<0.05$, $^{\ddagger}p<0.01$, data compared to the nonanaemic group.*

Table 1b. Patient characteristics.

	1. Nonanaemics	2. Iron deficiency	3. ACD
Disease activity			
ESR (< 10 mm/h)	36 (21-65)	57* (32-70)	87‡ (52-105)
CRP (< 6 mg/l)	23 (2-54)	20 (2-78)	35† (11-121)
C1qba (< 7 %)	8 (3-32)	19 (3-78)	25† (5-75)
Cytokines			
TNF (< 8 pg/ml)	14 (9-28)	8 (6-28)	33† (16-93)
IL-6 (< 10 pg/ml)	0 (0-20)	0 (0)	17* (0-82)

*Parameters of disease activity and TNF and IL-6 levels in patients with rheumatoid arthritis without anaemia (group 1), with iron deficiency anaemia (group 2), and with anaemia of chronic disease (ACD, group 3). Data are expressed as median with range. *$p<0.10$, †$p<0.05$, ‡$p<0.01$, data compared to the nonanaemic group.*

Transferrin microheterogeneity

In Fig. 1, examples are shown of a normal transferrin microheterogeneity pattern and of a pattern obtained from a RA patient with ACD. Percentages and concentrations of individual transferrin subfractions are given in Table 2 and 3. The 0-, 1- and 8-sialo transferrin isotypes were not detectable in control sera nor in patient sera. Fig. 2 shows the percentage distribution of transferrin subfractions and a shift in transferrin microheterogeneity is evident in all RA groups. This change is characterized by an increase of the percentage of highly sialylated transferrin fractions (% HSTf, $p<0.01$) and a decrease of the percentage 4-sialotransferrin (% 4-STf, $p<0.01$). The percentage of low sialylated transferrin fractions (% LSTf) is decreased in all groups, but only significantly in ACD ($p< 0.01$). Within the three RA groups, the % HSTf is higher ($p<0.01$) and the % 4-STf is lower in ACD ($p<0.05$) compared to nonanaemics. The % LSTf is lower in ACD ($p<0.01$) compared to both other groups.

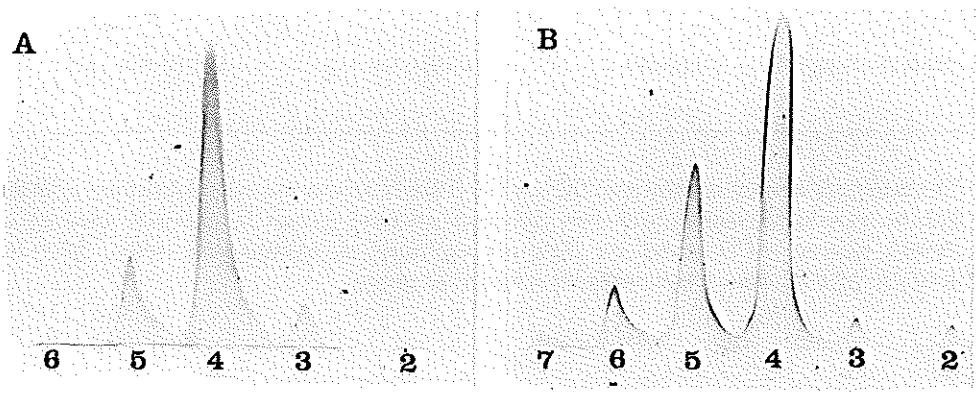

Figure 1.
Transferrin microheterogeneity patterns analyzed by crossed immuno isoelectric focusing. (A) Healthy individual. (B) Rheumatoid arthritis associated with the anaemia of chronic disease. The indices beneath the patterns indicate the number of sialic acids attached to the N-linked glycans of corresponding subfractions.

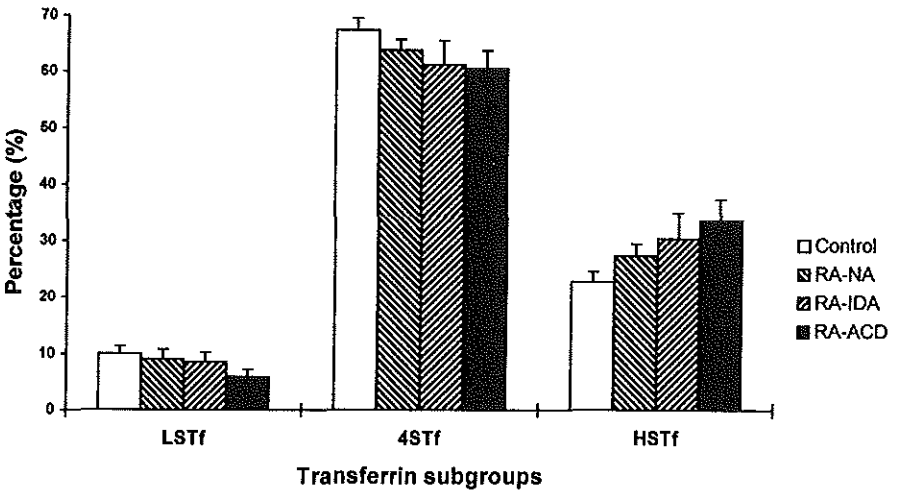

Figure 2. Percentual distribution of the transferrin subfractions in controls and patients with rheumatoid arthritis [RA]. NA, nonanaemics; IDA, iron deficiency anaemia; ACD, anaemia of chronic disease. % LSTf, % low sialylated transferrin fractions; % 4-STf, % 4-sialotransferrin; % HSTf, % highly sialylated transferrin fractions. Mean ± SD.

These data show that the shift to transferrins with highly branched glycans exhibits the same pattern in the three RA groups as the degree of disease activity. This shift is most pronounced in ACD, intermediate in iron deficiency anaemia and less pronounced in nonanaemics. This is illustrated by Table 4 which indicates that an increased inflammatory activity is associated with an increased % HSTf and a decreased % of 4-STf and LSTf. In addition, the percentual distribution of the transferrin subfractions correlates with the serum levels of TNF and IL6. Finally, an inverse relation is shown between the % HSTf and Hb, whereas the % 4-STf and LSTf correlate positively with Hb.

Table 2. Transferrin subfraction percentage in controls and patients with rheumatoid arthritis.

	2-sialo	3-sialo	4-sialo	5-sialo	6-sialo	7-sialo	Total
Controls	3 ± 0.5	8 ± 1	67 ± 2	18 ± 1	4 ± 1	0 ± 0	100 %
NA	3 ± 0.6	6 ± 1	64 ± 2	21 ± 2	6 ± 1	0 ± 0	100 %
IDA	2 ± 0.5	6 ± 2	61 ± 4	23 ± 3	7 ± 2	1 ± 0.4	100 %
ACD	2 ± 0.3	4 ± 1	60 ± 3	25 ± 2	8 ± 2	1 ± 0.4	100 %

NA, nonanaemics; IDA, iron deficiency anaemia; ACD, anaemia of chronic disease. Mean ± SD.

Table 3. Transferrin subfraction concentration (µmol/l) in controls and patients with rheumatoid arthritis.

	2-sialo	3-sialo	4-sialo	5-sialo	6-sialo	7-sialo	Total
Controls	0.9 ±0.2	2.9 ±0.5	25.7±2.4	6.8 ±1.2	1.7 ±0.3	0 ± 0	38.2±2.4
NA	0.7 ±0.2	1.9 ±0.6	18.4±4.0	6.1 ±1.4	1.6 ±0.4	0.1 ±0.1	28.9±6.2
IDA	0.7 ±0.2	1.9 ±0.7	19.3±3.8	7.1 ±0.9	2.1 ±0.4	0.2 ±0.1	31.4±4.5
ACD	0.5 ±0.1	0.9 ±0.3	14.6±2.5	6.0 ±1.1	1.9 ±0.5	0.3 ±0.1	24.1±3.9

NA, nonanaemics; IDA, iron deficiency anaemia; ACD, anaemia of chronic disease. Mean ± SD.

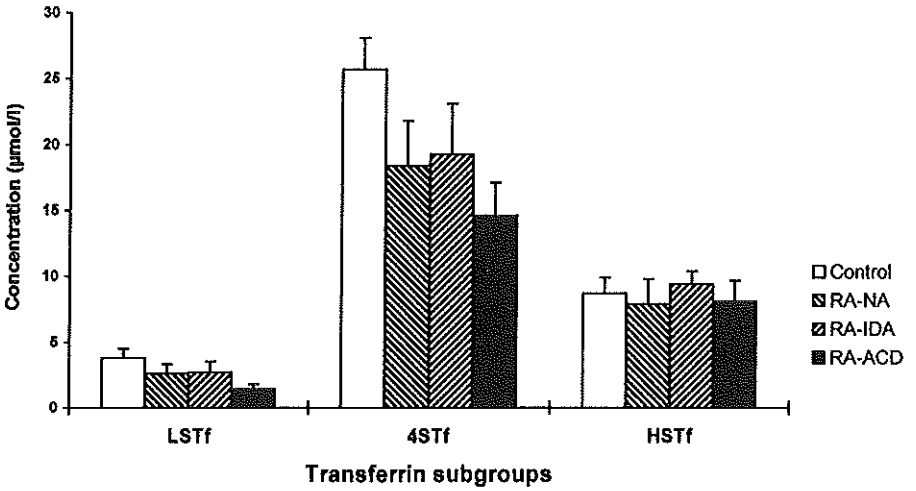

Figure 3. Transferrin subfraction concentrations in controls and patients with rheumatoid arthritis [RA]. NA, nonanaemics; IDA, iron deficiency anaemia; ACD, anaemia of chronic disease. LSTf, low sialylated transferrin fractions; 4-STf, 4-sialotransferrin; HSTf, highly sialylated transferrin fractions. Mean ± SD.

Transferrin subgroup concentrations are shown in Fig. 3. As total transferrin concentrations are reduced in all RA groups, transferrin subgroup levels, with exception of HSTf in group 2, are decreased as well. This involves predominantly the concentrations of LSTf and 4-STf which are decreased in all RA groups compared to controls ($p<0.01$). Within the three RA groups, LSTf and 4-STf levels are lower in ACD compared to nonanaemics and iron deficiency anaemia ($p<0.05$). No significant differences are observed in HSTf concentrations within the three RA groups or compared to controls.

Discussion

The aim of this study was to examine iron status and transferrin microheterogeneity in RA patients with and without ACD and to relate this to disease activity-related parameters and cytokines. It was shown that increasing inflammatory activity is associated with hypoferraemia elevated ferritin levels and a shift in the transferrin glycosylation pattern towards variants with highly branched glycan chains. These changes in iron metabolism were most pronounced in ACD and related to increased levels of TNF and IL-6.

Table 4. Correlations between transferrin subfraction distribution and Hb, disease activity and cytokines.

	% LSTf	% 4-STf	% HSTf
Hb	r = 0.69‡	r = 0.50*	r = -0.69‡
ESR	r = -0.73§	r = -0.52*	r = 0.74§
CRP	r = -0.61‡	r = -0.29	r = 0.49*
C1qba	r = -0.87‡	r = -0.55*	r = 0.75†
TNF	r = -0.41	r = -0.68‡	r = 0.65‡
IL-6	r = -0.33	r = -0.77‡	r = 0.79‡

% LSTf, % low sialylated transferrin fractions; % 4-STf, % 4-sialotransferrin; % HSTf, % highly sialylated transferrin fractions. $^{*}p<0.05$, $^{\dagger}p<0.01$, $^{\ddagger}p<0.005$, $^{\S}p<0.0005$.

ACD is a phenomenon of the chronic inflammatory state. This is illustrated by the observed higher disease activity in RA patients with ACD compared to patients without anaemia, confirming earlier reports (9). Not surprisingly, the development of ACD is related to release of proinflammatory cytokines (7,31), which may inhibit erythropoiesis and modulate iron metabolism. In this respect, TNF and IL-1, which both induce IL-6, may have an important role (7). ACD is characterized by profound changes in iron metabolism which limit erythroblast iron availability. First, this involves an increased iron retention by the liver and MPS, which may result from entrapment of labile iron into ferritin with a concomitant impaired iron release (10-12). In experimental animals, TNF and IL-6 induce hypoferraemia *in vivo* (32,33), whereas both mediators enhance ferritin synthesis *in vitro* (34,35). We found that serum levels of TNF and IL-6 correlate with serum iron and ferritin levels respectively, suggesting a role for these cytokines in the induction of hypoferraemia in ACD. Second, transferrin binding and iron uptake by erythroblasts may be decreased in ACD (13).

The observed shift in transferrin microheterogeneity in ACD may indicate preferential synthesis of transferrin fractions with highly branched glycan chains, since an increased glycan sialylation reflects, at least in part, an increased degree of glycan branching (15,36). Alterna-

tively, it could be argued that redistribution within the spectrum of transferrin isotypes results from an increased clearance of the partially desialylated variants, e.g. by the hepatic asialoglycoprotein receptor. However, in view of overall changes in plasma protein glycosylation in the inflammatory response, alterations in transferrin microheterogeneity in RA may primarily be based on modulation of transferrin glycosylation.

What are the consequences of the shift in transferrin microheterogeneity ? Generally, the carbohydrate moiety of plasma proteins is thought to influence intracellular protein transport, protein survival, protein-receptor interaction and specific biological activities (37). It has been shown that changes in glycosylation of glycoproteins can be accompanied by alterations in functional properties (22,38-40). During pregnancy, an increment of transferrins with highly branched glycans is thought to serve a functional purpose since it coincides with the increase in iron fluxes to both the placenta and the maternal bone marrow (14,15,17). In this concept, the increased synthesis of highly sialylated transferrin fractions in ACD may, in the face of both the impaired erythroblast iron availability in ACD and the counteractive decrease in total transferrin synthesis, be viewed as a compensatory mechanism in order to facilitate iron transport to erythroblasts. Several arguments, though, conflict with this hypothesis. First, incubation of human bone marow with ^{59}Fe-labelled 4-sialo and 6-sialotransferrin revealed no differences in iron uptake from both transferrin variants (41). Second, receptor binding of a transferrin variant lacking carbohydrates is unaffected, although iron uptake from this aglycotransferrin is reduced (42). Finally, considering the magnitude of changes in transferrin fractions it remains questionable whether these subtle alterations influence iron transport.

Another possible function of the shift in transferrin microheterogeneity involves protection from protein degradation. In the inflammatory state, expression of the hepatic asialoglycoprotein receptor may be upregulated (43) with a concomitant increased catabolism of low sialylated transferrins. This mechanism would then be counteracted by preferential synthesis of highly sialylated transferrins. Nonetheless, it is obvious that the (patho-)physiological significance of the shift in transferrin microheterogeneity has yet to be established.

One of the main characteristics of the acute phase response is the change in synthetic rates of plasma proteins (25). In addition, concomitant alterations are induced in the glycosylation of various positive APP, e.g. α1-acid glycoprotein and haptoglobin (19-24). It has been shown for numerous glycoproteins, including transferrin, that changes in the carbohydrate moiety are a concomitant feature of increased synthesis, and a linkage between the regulation of gene expression and glycosylation has been suggested (14,19). Transferrin, however, is a negative APP and this study shows that in active RA posttranslational modification may be directed towards increased synthesis of transferrin carrying highly branched glycans, under conditions

where transcription of the gene has diminished. This indicates that these processes are regulated by different mechanisms in the acute phase response. This is in accordance with the observed dissociation between the mechanisms which regulate the synthesis of α_1 -protease inhibitor and α- fetoprotein and their glycosylation (27,44).

TNF and IL6 levels were found to correlate with increased disease activity, which is in agreement with previous observations in RA (45,46). In the acute phase response, changes in both synthetic rate and carbohydrate moieties of positive APP are thought to be mediated by cytokines. In experimental animals and in primary cell cultures TNF, IL-1, IL6, and transforming growth factor ß1 have been shown to regulate gene expression and glycosylation of several positive APP (25-29). Of these cytokines, IL6 appears to be the most potent mediator (26). *In vitro* studies have shown that increased synthesis of CRP can be induced by IL6 but not by TNF (26). Our results are in concordance with this since, in contrast to TNF, IL-6 correlated positively with CRP. Both IL6 and TNF exert a negative influence on transferrin synthesis *in vitro* (25,26). The inverse relation between the levels of these cytokines and transferrin concentrations in this study would confirm a similar effect of IL6 and TNF on transferrin synthesis *in vivo* in active RA. It is not known whether the glycosylation of transferrin is affected by cytokines. The correlation between IL6 and TNF levels and the change in transferrin microheterogeneity found in this study, indicates that these mediators may influence transferrin glycosylation. It must be emphasized, though, that these correlations do not necessarily reflect a causal relationship. Therefore, in chapter 7 the effects of TNF administration are examined on transferrin glycosylation patterns in humans.

In conclusion, in RA the induction of both ACD and the shift in transferrin microheterogeneity is related to increased disease activity. Changes in transferrin synthesis and glycosylation are possibly mediated by differential effects of TNF and IL6. Although highly speculative, preferential synthesis of highly sialylated transferrins may play a role in the fine tuning of iron fluxes in ACD.

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Chapter 4

ERYTHROID TRANSFERRIN RECEPTOR EXPRESSION IN THE ANAEMIA OF CHRONIC DISEASE

Introduction

The course of chronic infectious, inflammatory and neoplastic disorders frequently coincides with the development of a mild and nonprogressive anaemia, originally described by Cartwright and Lee as anaemia of chronic disease [ACD] (1,2). Active rheumatoid arthritis [RA] is frequently accompanied by ACD and has often served as a model to study pathophysiological aspects of ACD (3,4). The pathogenesis of ACD is multifactorial and includes inhibition of erythropoiesis by cytokines like TNF, IL-1 and IFNγ (5-7), an impaired erythropoietin [EPO] response to the anaemia (8,9) and modulation of iron metabolism (10-14). Erythroblast iron availability is decreased in ACD, based on iron retention by liver and MPS with a concomitant induction of hypoferraemia (10-12). In addition, iron uptake by erythroblasts may be reduced, despite a decreased iron availability (13,14).

Iron incorporation into erythroblasts is thought to rely predominantly on transferrin receptor [TfR] mediated endocytosis (15,16). After binding of diferric transferrin, the transferrin-ligand complex is concentrated in coated pits and internalized (17,18). Acidification of endosomal compartments induces the release of transferrin-bound iron which can be incorporated into haeme (19-21). Subsequently, the receptor recycles to the cell surface and apotransferrin is released (20,21). The prime determinants of erythroblast iron availability are therefore the transferrin iron saturation, the amount of TfRs expressed by erythroblasts, the affinity of the TfR for its ligand and the kinetics of transferrin endo- and exocytosis.

It has been shown that both transferrin binding and iron uptake by erythroblasts *in vitro* are reduced in RA patients with ACD compared to controls or RA patients without anaemia or with iron deficiency anaemia (14). However, from these data it can not be differentiated whether this is based on an impaired TfR expression by erythroblasts in ACD or whether this is mediated by retention of radiolabelled iron by activated bone marrow macrophages. The aim of this study is therefore to examine the amount and affinity of TfRs on erythroblasts in RA patients with and without ACD.

Patients and Methods

Patients.

Serum and bone marrow from five healthy bone marrow donors and 14 patients with RA fitting the revised ARA-criteria (22) were studied. Group 1 comprised 5 healthy bone marrow transplantation donors, group 2 consisted of 5 nonanaemic RA patients (age: 57±4 years) and

group 3 of 9 patients with ACD (age: 66±6 years), based on a normal to increased amount of stainable bone marrow iron (23) and the exclusion of other causes of anaemia (see 2.1).

Laboratory procedures.

Erythrocyte parameters, iron status and disease activity

Determined were: Hb, Ht, reticulocytes, MCV, serum iron, transferrin, ferritin and stainable bone marrow iron (see 2.2, 2.3, 2.8). Serological disease activity was assessed by measurement of ESR and CRP levels (see 2.4).

Bone marrow studies

Bone marrow preparation, transferrin labeling and binding assays are described in detail in 2.8.2, 2.8.3 and 2.8.4. Briefly: a bone marrow cell suspension was layered over a Ficoll gradient in order to remove polymorphonuclear cells. Subsequently, total cell count, a differential count and cell viability, which was always higher than 85 %, were assessed.

Bone marrow mononuclear cells were incubated with ^{125}I-labelled diferric transferrin in a concentration range of 0.02 nmol/ml to 2 nmol/ml at 0 °C for 60 min. After four washing steps radioactivity of the cell bound ligand was measured using a Packard-autogamma 500.C. (24). Non-specific binding, usually less than 10 %, was determined by measuring the radioactivity bound to the cells in the presence of a 100-fold excess of unlabelled transferrin. The association constant (Ka) and the number of TfRs per erythroblast were calculated by the method of Scatchard (25).

Results

Iron status and disease activity

Patient characteristics are shown in Table 1. In comparison with nonanaemic RA patients serum iron concentrations are lower in RA patients with ACD ($p<0.05$). Serum transferrin levels are also lower in ACD, although not significant. Serum ferritin concentrations are elevated in RA patients with ACD compared to nonanaemics ($p<0.01$).

Serological disease activity, as assessed by ESR and CRP, is higher in ACD compared to nonanaemic patients ($p<0.05$).

Table 1. Patient characteristics.

	Nonanaemics	ACD
Erythocyte parameters		
Hb (7.4-10.9 mmol/l)	8.0 (7.7-8.3)	6.1 (5.5-7.2)
Ht (0.36-0.51 l/l)	0.39 (0.38-0.42)	0.33 (0.28-0.36)
MCV (80-96 fl)	87 (84-102)	83 (66-90)
Iron status		
Iron (14-30 µmol/l)	9 (5-15)	6* (1-10)
Transferrin (25-55 µmol/l)	64 (47-78)	45 (40-59)
Ferritin (20-150 µg/l)	32 (10-326)	165† (73-250)
Disease activity		
ESR (< 10 mm/h)	34 (21-46)	54* (34-118)
CRP (<6 mg/l)	6 (4-25)	61* (5-104)

*Erythrocyte variables, iron status and parameters of disease activity in patients with rheumatoid arthritis without anaemia (group 1) and with anaemia of chronic disease (ACD, group 2). Data are expressed as median with range. *$p<0.05$, †$p<0.01$, data compared to the nonanaemic group.*

Transferrin receptor number and affinity

Scatchard analysis of ^{125}I-transferrin binding to erythroblasts revealed that the number of transferrin binding sites per cell in the three groups are (data expressed as median with range): 118000 (80800-130000) in controls, 102000 (72000-129000) in nonanaemic RA patients and 74400 (53100-122000) in RA patients with ACD (see Fig. 1 and Fig. 2). The number of TfRs on erythroblasts is reduced in ACD compared to nonanaemic RA patients ($p<0.05$) and controls ($p<0.02$).

The Ka values (1/M), which represent the affinity of the TfR, in the three groups are (data expressed as median with range): 1.3 $.10^7$ (0.2-6.5) in controls, 0.8 $.10^7$ (0.2-1.8) in nonanaemic RA patients and 0.5 $.10^7$ (0.3-6.3) in RA patients with ACD. Receptor affinity did not differ significantly between the three groups, but tends to be lower in ACD (see Fig. 2).

Bone marrow differential counts did not show significant differences in number or maturity of erythroblasts between the three groups. The mean percentages of erythroblasts in bone marrow mononuclear cells were: 32 % in controls, 37% in the nonanaemic RA patients and 31 % in RA patients with ACD.

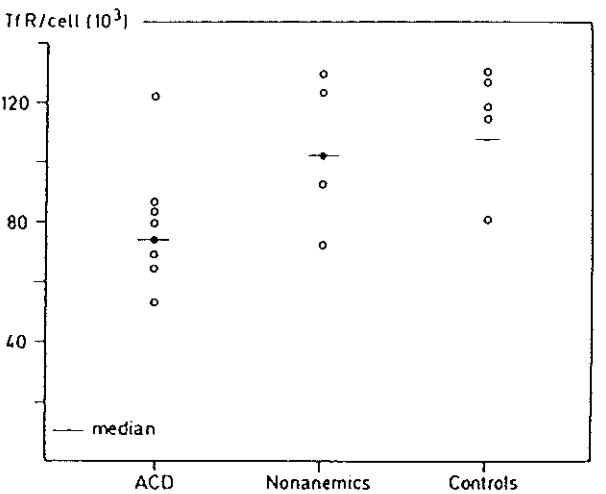

Figure 1.
Transferrin receptor expression in rheumatoid arthritis (RA). Number of transferrin receptors (TfR) per bone marrow mononuclear cell in controls, nonanaemic RA patients and RA patients with the anemia of chronic disease (ACD). Significant differences are described in the text (results).

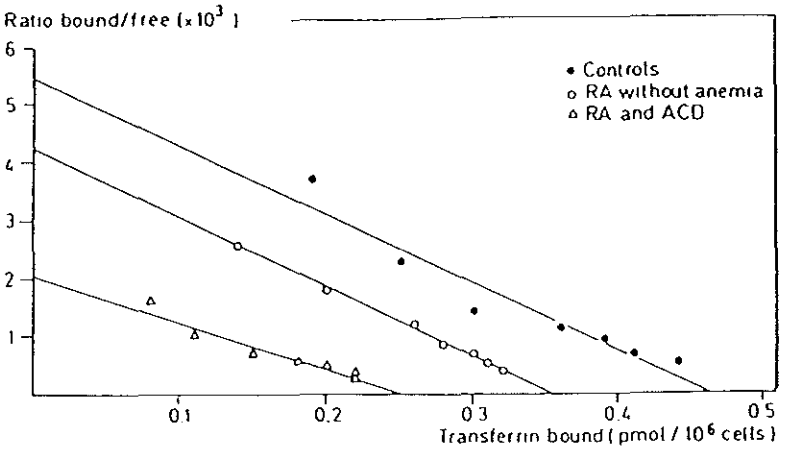

Figure 2. Scatchard analysis of transferrin binding on bone marrow mononuclear cells from patients with rheumatoid arthritis with and without the anaemia of chronic disease (ACD) and a healthy control. Bound/Free is the ratio of ^{125}I-transferrin bound to the cells and free ^{125}I-transferrin.

Discussion

Receptor mediated endocytosis of diferric transferrin is thought to be the predominant mode of iron incorporation into erythroblasts (15,16). During erythropoiesis the rate of iron uptake at various stages is closely correlated to the number of expressed TfRs. Receptor affinity and the rate of transferrin endo- and exocytosis, however, remain unchanged (26). In addition, in iron deficiency anaemia increased iron uptake by erythroblasts is related to an increased number of surface TfRs, since affinity and transferrin kinetics are not modulated (27). These data indicate that the degree of TfR expression is an important regulatory factor of erythroid iron uptake (26,27). Previously, bone marrow incubation studies with ^{59}Fe-labelled ^{125}I-transferrin *in vitro* revealed that both transferrin binding and iron uptake by erythroblasts are reduced in RA patients with ACD (14). Theoretically, this might be based on: (a) an intrinsic defect in erythroblast iron uptake capacity; (b) inhibition of transferrin binding and iron uptake; (c) retention of iron by activated bone marrow macrophages. These mechanisms will be discussed separately in the context of this study.

The present data imply that the observed reduction of transferrin binding and iron incorporation in ACD might rely on an impaired erythroid TfR expression. In addition, the

affinity of the TfR was slightly decreased in ACD, although it is questionable whether this significantly affects transferrin binding and iron uptake. The affinity of the TfR is thought to be influenced by its carbohydrate moiety which is attached to the extracellular domain (28). In acute and chronic inflammatory states major changes occur in the glycosylation of numerous glycoproteins (29). It might therefore be speculated that in ACD alterations are induced in the carbohydrate moiety of the TfR as well, with a concomitant modulation of its affinity. This needs, however, further investigation. Several reports indicate that the intracellular processing of transferrin-bound iron may also influence cellular iron uptake, since several pathways appear to be involved in iron removal from transferrin and intracellular iron transport (30). For instance, in erythroleukemia, iron uptake by erythroblasts may be decreased based on insufficient iron removal from endocytosed transferrin (31). Whether alterations in the intracellular release and transport of iron are operative in ACD is presently unknown.

Apart from a decreased number and affinity of TfRs, the reduced iron uptake in ACD may in part be explained by inhibition of transferrin binding. *In vitro*, the acute phase protein α1-antitrypsin inhibits transferrin binding and internalization by erythroid precursors in a dose-dependent and competitive manner (32). This effect could be demonstrated at physiological concentrations, maximum inhibition, though, was attained at α1-antitrypsin concentrations as achieved in the acute phase response. Other acute phase proteins like CRP, haptoglobin and ferritin and the cytokines TNF, IL-1 and IL-6 did not affect the interaction between transferrin and its receptor. In addition, α1-antitrypsin suppresses erythroid growth, presumably by interfering with cellular iron uptake. Interestingly, α1-antitrypsin is not only produced by hepatocytes, but also by macrophages (33). The activated bone marrow macrophage is postulated to have a key role in the suppression of erythropoiesis in ACD (34), which may thus be mediated via secretion of α1-antitrypsin, next to release of inhibitory cytokines.

It could be argued that erythroid transferrin binding and iron uptake are reduced in ACD based on preferential transferrin uptake and iron retention by activated bone marrow macrophages, which indeed have an increased ferritin content in ACD (11). However, this seems a less plausible explanation considering differences in surface TfR number on erythroblasts versus macrophages (35). In addition, *in vitro* studies on transferrin-iron uptake by inflammatory macrophages show conflicting results (36,37). In this study we did not measure the magnitude of ^{125}I-transferrin binding to non-erythroid mononuclear cells. The assumption that erythroblasts mainly account for ^{125}I-transferrin binding is based on studies demonstrating that TfRs are predominantly expressed by erythroblasts and that the amount of ^{125}I-transferrin binding closely correlates with the number of erythroblasts (27,38). Thus, although it can not fully be excluded that a small amount of ^{125}I-transferrin has bound to other

mononuclear cells, our data are likely to reflect the TfR status on erythroblasts in ACD. This is confirmed by the study of Kuiper-Kramer et al. who assessed erythroid TfR expression using flow cytometry and also found a decreased number of TfRs on erythroblasts in ACD (39).

How could erythroid TfR expression be down-regulated in ACD ? The synthesis of TfRs is regulated at post-transcriptional level by the intracellular iron concentration which modulates the interaction of iron regulatory proteins [IRP] with an iron responsive element [IRE] of the TfR mRNA. As a result, a decrease in intracellular iron induces stability of TfR mRNA which results in an increased TfR synthesis and vice versa (40,41). Increased iron retention by MPS and liver, reflected in hypoferraemia and increased ferritin levels, is thought to reduce erythroblast iron availability in ACD (10-12). In the IRP-IRE-concept this would result in an up-regulation of TfR synthesis. We found, however, a decreased TfR expression in ACD.

First, this might be mediated by inhibitory effects of cytokines. In ACD, erythropoiesis is impaired as illustrated by a decreased growth *in vitro* of erythroid burst- and colony forming units [BFU-E and CFU-E], representing early and late erythroid progenitors respectively (42,43). However, the number of late erythroid precursors in RA patients with and without ACD appeared to be similar (44). This might point to a rather functional disturbance of erythroid proliferation in ACD. Cytokines like TNF, IL-1 and IFNγ suppress human BFU-E and CFU-E proliferation *in vitro* (6,7,43,45), although the exact mechanism has not been clarified yet. It might be speculated that this is mediated, in part, through inhibition of erythroid TfR expression resulting in cellular iron deprivation. For instance, TNF decreases ^{59}Fe-incorporation by erythroid precursors in rats (13), possibly via a reduction in TfRs. *In vitro*, IFNγ inhibits TfR synthesis at post-transcriptional level, with a concomitant impaired cell growth (46). It must be emphasized, though, that a decreased erythroid TfR expression in ACD may be a consequence rather than a primary cause of an impaired erythropoiesis. Further study is needed to examine the mechanisms of cytokine-mediated inhibition of erythropoiesis in ACD and the effects of cytokines on cellular iron homeostasis.

Second, the reduction in erythroid TfR number may be related to an impaired EPO responsiveness in ACD. EPO is an important growth factor for erythropoiesis and is able to stimulate TfR expression (47,48). However, in ACD EPO levels are inappropriately low for the degree of anaemia (8,9), which may thus contribute to a decreased erythroid TfR expression in ACD. This is supported by the observed rise in serum TfR levels in RA patients with ACD following treatment with recombinant human EPO (see chapter 5). In addition, the response of erythroid precursors, and thus TfR expression, to EPO may be decreased in ACD (49), possibly mediated by cytokines. Indeed, inhibition of erythroid colony growth by TNF or IFNγ *in vitro* could be counteracted by exposure to high EPO concentrations (45,50).

The failure of ACD to respond to iron administration (3) may be explained by the direction of iron fluxes toward storage sites and the decreased erythroid TfR expression and iron uptake. The beneficial effects of iron chelators in ACD in RA (51), however, may partly be related to an increased TfR expression since iron chelation is thought to upregulate the number of transferrin binding sites on erythroblasts (52,53).

In conclusion, these preliminary data demonstrate that in RA accompanied by ACD TfR expression by erythroblasts is significantly reduced. This might underlie the observed decrease in both transferrin binding and iron incorporation in ACD, since the number of transferrin binding sites is the major determinant of erythroid iron uptake.

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Chapter 5

SERUM TRANSFERRIN RECEPTOR LEVELS IN THE ANAEMIA OF CHRONIC DISEASE

Diagnostic significance and effects of recombinant human erythropoietin and iron chelation treatment

Introduction

Active rheumatoid arthritis [RA] is frequently accompanied by anaemia, which can be of different origins (1,2). This involves predominantly iron deficiency anaemia [IDA] and the anaemia of chronic disease [ACD] (3-6). The differentiation between these types of anaemia is a common diagnostic problem, since parameters as serum iron, total iron binding capacity, transferrin iron saturation, mean corpuscular volume, red cell distribution width and erythrocyte ferritin often lack adequate sensitivity and specificity to distinguish between IDA and ACD (4,7,8). Serum ferritin is a reliable marker of iron deficiency (4). However, apart from body iron stores, the ferritin concentration is influenced by the acute phase response (4,9). In RA an increased disease activity is associated with elevated ferritin levels and the induction of ACD (4,10). It can be difficult to exclude co-existing iron deficiency [ID] in this condition and therefore stainable bone marrow iron remains the gold standard to assess body iron stores.

With the introduction of the serum-soluble transferrin receptor [sTfR] assay a new method is available to detect ID (11-14). It has been shown that sTfR levels are related to tissue TfR levels and the rate of erythropoiesis (11,14-16). In IDA, in which erythroid TfR expression is up-regulated, high sTfR levels are found (11-14). Ferguson et al. have demonstrated that the sTfR assay may be a sensitive method to distinguish ACD from IDA (17). In this study a significant rise in sTfR levels was observed in IDA, whereas normal sTfR levels were found in ACD. The ACD subjects, however, were heterogeneous with respect to the underlying disorder. It is therefore not known whether the sTfR concentration can differentiate both types of anaemia within one entity such as RA.

The pathogenesis of ACD is multifactorial and the following mechanisms are thought to be involved: (a) inhibition of erythropoiesis by cytokines (18-20), (b) an impaired erythropoietin [EPO] response to the anaemia (2,10,21) and (c) a decreased erythroblast iron availability resulting from an increased iron retention by liver and MPS (22,23) and a decreased iron uptake by erythroblasts (24), which might rely on an impaired erythroid TfR expression as shown in chapter 4. Based on these pathophysiological mechanisms trials have been carried out in which RA patients with ACD were treated with recombinant human erythropoietin [r-HuEPO] and the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one [L1] respectively. Both treatment modalities appeared to improve erythropoiesis (25-27).

The aim of this study is to examine the relation between sTfR levels and iron stores in RA patients with and without anaemia as well as the effects of treatment of RA patients with ACD with r-HuEPO and iron chelation on sTfR levels and iron status.

Chapter 5

Patients and Methods

Patients

(a) Untreated patients: 9 RA patients without anaemia, 9 RA patients with IDA and 11 RA patients with ACD were included. IDA was diagnosed based on the absence of stainable bone marrow iron content, whereas ACD was defined by a normal to increased amount of stainable bone marrow iron and the exclusion of other causes of anaemia (see 2.1 and 2.8.1). Age, sex and disease duration in the three groups were comparable.

(b) Treatment with r-HuEPO: 10 RA patients with ACD were treated with r-HuEPO (Boehringer, Mannheim, Germany) for 6 weeks in a dose of 250 U/kg subcutaneously, 3 times a week (26). STfR levels, parameters of erythropoiesis and iron status were determined before and after 3 and 6 weeks of treatment respectively.

(c) Treatment with iron chelation: 6 RA patients with ACD were treated with the oral iron chelator L1 for 3 weeks using a dose of 3 grams daily (27). sTfR levels, parameters of erythropoiesis and iron status were compared before and after treatment.

Laboratory Procedures

Determined were: Hb, Ht, reticulocyte count, CRP, serum iron, transferrin and ferritin and bone marrow iron content (see 2.2-2.4, 2.8).

sTfR concentrations were measured using a modified ELISA using two non-cross-reactive monoclonal antibodies, OKT 9 and J 64 (see 2.9). In 20 healthy blood donors median sTfR was 2.8 (1.7-3.5) mg/l.

Results

Untreated patients

Data are shown in Table 1 and Fig 1. Hb levels were comparable in RA patients with ACD and IDA. CRP levels were higher in RA patients with ACD ($p<0.05$) compared to nonanaemic and iron deficient RA patients. Serum ferritin levels were below 50 µg/l in IDA, except for one patient. In ACD, serum ferritin levels were above 50 µg/l and were increased compared to RA patients without anaemia or with IDA ($p<0.05$). sTfR values were similar in RA patients without anaemia and with ACD and slightly increased compared to controls (not significant). In RA patients with IDA sTfR levels were elevated as compared to controls, nonanaemics and RA patients with ACD ($p<0.05$). sTfR levels correlated with Hb values in IDA ($r=-0.90$, $p<0.05$). No correlation was found between sTfR levels and ferritin levels.

Table 1. Patient characteristics in RA patients without anaemia and with ACD or iron deficiency anaemia.

	Nonanaemics	ACD	Iron deficiency
Hb (7.4-10.9 mmol/l)	8.1 (7.4-8.7)	6.3 (5.7-6.9)	6.4 (4.3-7.1)
CRP (< 6 mg/l)	6 (3-29)	71* (7-110)	20 (5-67)
Ferritin (20-150 µg/l)	33 (10-326)	121* (50-432)	15 (10-53)
sTfR (1.7-3.5 mg/l)	4.3 (2.8-6.9)	4.3 (2.3-6.1)	6.3* (4.8-10.3)

*Hb, CRP, ferritin and serum transferrin receptor [sTfR] levels in patients with rheumatoid arthritis [RA] without anaemia and with anaemia of chronic disease [ACD] or iron deficiency anaemia. Data are expressed as median with range. *$p<0.05$.*

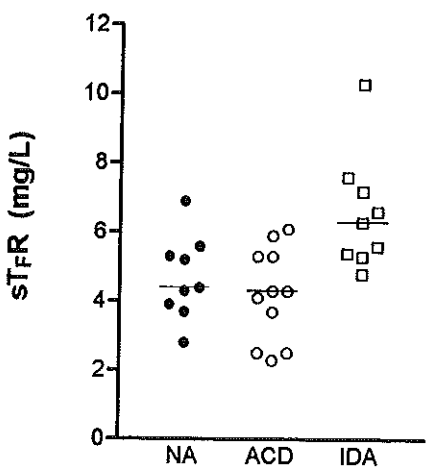

Figure 1. Serum transferrin receptor [sTfR] levels in patients with rheumatoid arthritis without anaemia [NA] and with anaemia of chronic disease [ACD] or iron deficiency anaemia [IDA]. Horizontal bars represent median values.

Treatment with r-HuEPO

Data are shown in Table 2 and Fig.2. During treatment with r-HuEPO Hb levels and reticulocyte count increased significantly at 3 and 6 weeks. sTfR levels before treatment were higher compared to healthy controls ($p<0.01$). Treatment with r-HuEPO induced an increase in sTfR levels ($p<0.001$) and a decrease in ferritin levels ($p<0.01$) as measured at 3 and 6 weeks. ID, defined by ferritin levels below 50 µg/l (4), occurred in 2 patients at 3 weeks and in 5 patients at 6 weeks. sTfR levels in ID patients at 6 weeks were slightly higher (10.6, range 7.6-12.7 µg/l) compared to patients without ID (8.9, range 5.7-10.2 µg/l, not significant). Serum iron levels did not change significantly during r-HuEPO treatment.

Table 2. Characteristics in RA patients with ACD before and after treatment with r-HuEPO.

	Baseline	3 weeks	6 weeks
Hb (7.4-10.9 mmol/l)	5.9 (5.5-7.0)	6.7* (5.8-7.8)	7.2† (5.9-8.5)
Reticulocytes	18 (2-33)	43† (27-52)	34* (6-58)
Iron (14-30 µmol/l)	5 (1-9)	5 (1-10)	4 (1-9)
Ferritin (20-150 µg/l)	180 (139-318)	118† (11-301)	37‡ (9-157)
sTfR (1.7-3.5 mg/l)	4.0 (2.3-5.1)	7.8‡ (4.1-10.5)	9.6‡ (5.7-12.7)

*Hb, serum iron, ferritin and serum transferrin receptor [sTfR] levels in patients with rheumatoid arthritis [RA] and anaemia of chronic disease [ACD] during treatment with recombinant human erythropoietin [r-HuEPO]. Data are expressed as median with range. *$p<0.05$, †$p<0.01$, ‡$p<0.001$.*

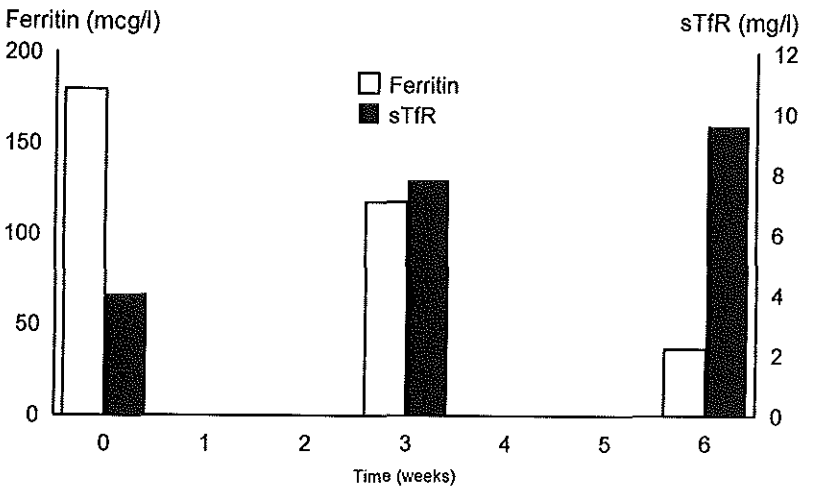

Figure 2. Serum ferritin and serum transferrin receptor [sTfR] levels during treatment with r-HuEPO in patients with rheumatoid arthritis and the anaemia of chronic disease.

Table 3. Characteristics in RA patients with ACD before and after treatment with L1.

	Baseline	**3 weeks**
Hb (7.4-10.9 mmol/l)	6.3 (5.9-7.0)	6.7* (5.6-7.3)
Iron (14-30 µmol/l)	6 (4-11)	12† (7-21)
Ferritin (20-150 µg/l)	118 (40-269)	78* (10-191)
sTfR (1.7-3.5 mg/l)	4.7 (2.3-6.1)	6.2† (2.9-9.2)

*Hb, serum iron, ferritin and serum transferrin receptor [sTfR] levels in patients with rheumatoid arthritis [RA] and anaemia of chronic disease [ACD] before and after 3 weeks of L1 treatment. Data are expressed as median with range. *$p<0.10$, †$p<0.05$.*

Treatment with L1

Data are shown in Table 3 and Fig. 3. Treatment with L1 resulted in a significant increase in Hb levels. Serum ferritin levels decreased, whereas serum iron levels increased during iron chelation treatment. Baseline sTfR concentrations were slightly higher compared to controls (not significant). A moderate increase in sTfR levels ($p<0.05$) was observed after 3 weeks of L1 treatment.

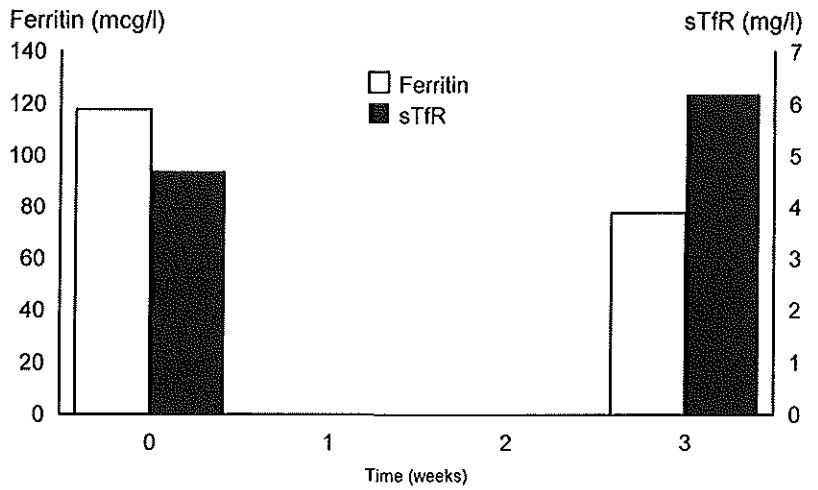

Figure 3. Serum ferritin and serum transferrin receptor [sTfR] levels before and after treatment with L1 in patients with rheumatoid arthritis and the anaemia of chronic disease.

Discussion

General aspects

The TfR is a transmembrane glycoprotein predominantly expressed by erythroid progenitor cells (28). Cellular iron uptake relies on receptor mediated endocytosis. Diferric transferrin binds to the TfR followed by endocytosis of the receptor-ligand complex. After the release of transferrin-bound iron, the receptor-ligand complex recycles back to the cell surface, followed by dissociation of apotransferrin from the TfR (29,30). As a result of externalization of TfRs during the endocytic cycle, a soluble form of TfR can be detected in serum, which appears to be a 85 kDa monomeric fragment of the extracellular domain (14-16)

Several reports show the existence of a constant relationship between membrane receptors and plasma receptors (11,16). This implies that the mechanisms which modulate TfR expression determine sTfR levels. The synthesis of TfRs is controlled at post-transcriptional level by the intracellular iron concentration (31). TfR turnover is upregulated in IDA and during an increased rate of erythropoiesis, governed by the action of EPO, which stimulates erythroid growth and TfR expression (28,32). In IDA, elevated sTfR levels correlate with EPO and ferritin levels (16,17). In addition, a close correlation was found between sTfR levels and erythron transferrin uptake, a ferrokinetic parameter associated with the rate of erythropoiesis, in both healthy individuals and patients with various haematologic disorders (11,33). Thus, sTfR levels reflect tissue iron stores and erythroid proliferation.

Diagnostic significance of the sTfR

The present data show that in RA sTfR concentrations are significantly elevated in patients with IDA, compared to nonanaemics and ACD. This is in line with the study of Punnonen et al. who also found increased sTfR levels in anaemic patients with chronic disorders without stainable bone marrow iron (34). However, sTfR levels in RA-IDA were not as high as measured in IDA without an accompanying inflammatory disorder, in which an increase of about 3 times normal has been reported (16,17). This might be explained by differences in TfR expression related to an altered EPO response to the anaemia. Indeed, in RA patients with depleted iron stores serum EPO levels are higher than in those without anaemia or with ACD, but lower than in an "uncomplicated" IDA (2,17,21). In patients with ACD it can be difficult to detect co-existing ID by means of serum ferritin since its concentration is influenced by the acute phase response, resulting in disproportional high ferritin levels in relation to iron stores (4,9). This diagnostic problem can partly be met by increasing ferritin cut-off levels for ID to values between 50 and 100 µg/l (2,3,35,36). In this study ID could be identified using a cut-off point of 50 µg/l. However, other studies show that ferritin levels above the adapted cut-off points do not fully exclude co-existing ID (35,36). The use of this approach in clinical practice is further limited due to lack of standardization. Measurement of sTfR levels may be an additional diagnostic tool in the assessment of iron stores in inflammatory states. First, sTfR levels are a sensitive marker of depletion of iron stores since elevated sTfR levels are found in early ID without anaemia (12). Second, compared to serum ferritin sTfR levels may have the advantage of being less sensitive to disease activity. Larger studies in patients with disorders of various etiologies as well as standardization of the sTfR assay are needed to confirm the diagnostic significance of sTfR concentrations in the detection of ID in ACD.

Relation with erythroid TfR expression

In ACD iron is redistributed to storage compartments (22,23) which results in a functional ID of bone marrow as reflected by a decreased number of sideroblasts (6). In physiological conditions this would be counteracted by a compensatory increase in erythroid TfR expression with a concomitant rise in sTfR levels. However, *in vitro* iron uptake and transferrin binding by erythroblasts appear to be decreased in ACD (24). In chapter 4 it was shown that this might rely on an impaired erythroid TfR expression. Consequently, a decrease in sTfR levels is likely to occur in ACD. Indeed, an experimental animal study has demonstrated a reduction in sTfR values during inflammation (37). We found normal to slightly elevated sTfR levels in ACD which is in accordance with the study of Ferguson et al. (17). Kuiper-Kramer et al. also found normal sTfR concentrations in ACD, whereas the number of TfRs on erythroid precursors, which were simultaneously measured, appeared to be decreased (38). Thus, in the face of a reduced erythroblast iron availability sTfR levels are relatively low in ACD and may reflect an impaired erythropoiesis and TfR turnover. However, some discrepancy remains between *in vitro* erythroid TfR expression (normal to decreased) and peripheral sTfR levels (normal to increased).

First, this could be explained by an increased rate of TfR shedding in ACD, possibly in the context of inhibition of erythroid iron uptake. Second, sTfR clearance might be decreased in ACD, although the mechanism of sTfR catabolism is currently unknown. Third, TfRs derived from nonerythroid cell types may affect sTfR levels in ACD. For instance, activation of T-lymphocytes, frequently occuring in conditions associated with ACD, is accompanied by an increase in TfR expression in order to facilitate the growth-promoting effects of transferrin (39,40). This could, in turn, increase sTfR levels in ACD. Further study is, of course, needed to substantiate these possibilities.

Effects of r-HuEPO treatment

Treatment of RA patients with ACD with r-HuEPO resulted in an improvement of erythropoiesis as evidenced by an increase in Hb levels and reticulocyte count. This confirms that an impaired EPO responsiveness plays a role in the pathogenesis of ACD. We observed a significant increase in sTfR levels during treatment with r-HuEPO. First, this might be based on an increased erythroid precursor cell mass and an upregulated erythroid TfR expression induced by r-HuEPO. Second, the rise in sTfR levels may be related to depletion of iron stores or even ID. Administration of r-HuEPO resulted in iron mobilization from storage sites reflected by a decrease in ferritin levels. In the patients who became ID during r-HuEPO treatment sTfR levels were slightly higher. The iron mobilizing effects of r-HuEPO may be explained by an

increased iron utilization by erythroblasts. In addition, r-HuEPO might have a direct effect on iron release from iron stores. The mechanisms which regulate iron donation by the MPS and hepatocytes to transferrin are not clear. Macrophages may express receptor sites for EPO (41), it might therefore be speculated that EPO directly enhances iron release from storage sites. Thus, during r-HuEPO treatment sTfR levels may be used to monitor erythropoiesis and, in combination with serum ferritin, to detect development of ID.

Effects of iron chelation treatment

Iron chelation treatment with L1 of RA patients with ACD resulted in a slight increase in Hb values accompanied by a decrease in ferritin and an increase in sTfR levels. These preliminary results suggest that iron chelation may improve erythropoiesis in ACD. First, this might be explained by an increase in erythroblast iron availability. L1 appears to be able to release iron from the storage compartment, reflected by a decrease in serum ferritin levels. Indeed, L1 enhances iron release by macrophages *in vitro* (42). Subsequently, iron transport to the bone marrow may be facilitated by L1 (43) or by transfer of L1-bound iron to transferrin (44). In addition, iron chelation may improve erythroblast iron availability by increasing erythroid TfR expression as has been demonstrated by *in vitro* studies (45,46). Second, increased endogenous EPO levels may contribute to the rise in Hb levels during iron chelation treatment. EPO levels correlate negatively with iron stores in ACD and in a pilot study with L1 in RA patients with ACD a rise in EPO levels was observed after one week of treatment (47). This points to an inhibitory effect of iron stores on EPO responsiveness in ACD. The increase in sTfR levels following iron chelation treatment might thus be explained by depletion of tissue iron stores and an increased erythroid proliferation secondary to elevated EPO levels and an improved iron availability.

In conclusion, the sTfR may be a useful parameter to assess iron stores in RA. A definitive place for the sTfR assay in the detection of co-existing ID in chronic inflammatory disorders has to be established in larger studies. Treatment of RA patients with ACD with r-HuEPO and L1 respectively improves erythropoiesis and is accompanied by a rise in sTfR levels reflecting an increased erythroid proliferation and depletion of iron stores.

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Part II

IRON METABOLISM AND ENDOCRINE SYSTEMS IN THE ACUTE PHASE RESPONSE

TNF administration to cancer patients as model for the acute inflammatory state

Chapter 6

REGULATORY ASPECTS OF THE ACUTE PHASE RESPONSE

Effects of tumor necrosis factor α administration on cytokines, cytokine receptors and acute phase proteins in cancer patients

Introduction

The acute phase response refers to a coordinated series of physiological changes in response to tissue injury (1,2). Stimuli which initiate the acute phase response can be of infectious, inflammatory, neoplastic or traumatic origin. One of the main features of the acute phase response is the modulation of hepatic plasma protein synthesis (1,2). This involves a sequential increase in production of (positive) acute phase proteins [APP's], whereas the synthesis of another subset of (negative) APP's decreases. Apart from modulation of APP synthesis, changes occur in post-translational modification of most APP's resulting in an altered glycosylation pattern (3,4). APP's are thought to have a key role in tissue repair processes and restoring of homeostasis.

The initiation and coordination of the APR is primarily regulated by cytokines, which are operating in a complex network (2,5). TNF is a proximal mediator within this network and exerts a wide spectrum of biological activities in which it acts synergistically with IL-1 (5-7). Both TNF and IL-1 are predominantly produced by activated macrophages (5-7), which are primed by the T-cell derived cytokine IFNγ (8). TNF and IL-1 initiate a cascade of additonal mediators like IL-6, a multifunctional cytokine (9-11). Cytokines regulate APP synthesis as has been shown by *in vitro* studies with rat hepatocytes, human hepatoma cell lines and primary human hepatocytes (1,2,12-15). *In vivo*, administration of TNF, IL-1 or IL-6 to rodents induces alterations in hepatic mRNA concentrations of both positive and negative APP's (14,15). Little is known, however, about the *in vivo* effects of cytokines on APP production in humans. In chapter 3 we found a correlation between serum levels of IL-6 and CRP in RA patients, although this implies not necessarily a causal relationship.

The proinflammatory cytokine cascade is antagonized by several counterregulatory mechanisms. One of these mechanisms involves the release of soluble cytokine receptors, e.g. for TNF and IL-1. The TNF receptor exists in two forms with a molecular mass of 55 and 75 kDa respectively (16). Shedding of the extracellular domain of these TNF receptors by proteolytic cleavage results in circulating soluble TNF receptors, termed sTNF-R55 and sTNF-R75 (17,18). Soluble TNF receptors are constitutively produced (19) and elevated sTNF-R55 and sTNF-R75 concentrations are found in infectious, inflammatory and malignant disorders (20-22) as well as in renal failure (23). Both sTNF-R55 and sTNF-R75 have been postulated to act as TNF antagonists by binding and inactivation of circulating TNF (24).

Isolated limb perfusion [ILP] with high-dose recombinant human TNF [r-HuTNF], recombinant human IFNγ [r-HuIFNγ] and melphalan is an effective treatment for patients with inoperable melanoma or sarcoma (25,26). After this procedure, when the limb is reconnected to the systemic circulation, resorption of residual TNF from the perfused limb results in high systemic

TNF levels, providing a model to examine *in vivo* effects of TNF in the acute inflammatory state (27). To obtain more insight in the regulation of the acute phase response we examined the prolonged effects of r-HuTNF administration to cancer patients on serum concentrations of TNF, soluble TNF receptors, IL-6 and APP's.

In chapter 7, this subset of patients is studied with respect to the effects of cytokine administration on iron metabolism in the acute phase response. In chapter 8, the effects of TNF infusion on endocrine aspects of the inflammatory response, in particular changes in thyroid hormone and cortisol metabolism, are examined.

Patients and Methods

Patients

Twelve patients were studied with irresectable soft tissue sarcoma (n=8) or melanoma with multiple in transit melanoma metastases (n=4). Median age was 59 years (range 29-78 years). Six patients were male, six were female.

Isolated limb perfusion

The method of ILP is described in 2.10. In short, patients are pretreated daily with 0.2 mg r-HuIFNγ subcutaneously on the two days before ILP. The ILP procedure is carried out under general anaesthesia. After cannulation of the local vasculature, the limb is perfused via an extracorporeal circuit during 90 minutes with r-HuTNF (2 mg/arm or 4mg/leg) and 0.2 mg r-HuIFNγ. Melphalan (13 mg/l arm volume or 10 mg/l leg volume) is administrated after 30 minutes. ILP is followed by drainage of the perfusate from the limb and a washout procedure. After the ILP procedure patients are extubated and observed at the intensive care unit for 24 hours.

Blood sampling

Before ILP, blood samples were obtained on the two pretreatment days (day -2, day -1) and at baseline. After the end of the ILP procedure, defined as the moment of releasing the tourniquet with reconnection of the limb to the systemic circulation, serum was collected at 1, 3, 5, 10 and 30 minutes [min] and 4 and 8 hours [h]. Subsequently, serum was obtained on 7 consecutive days after ILP at 8.00 h.

Laboratory procedures

Cytokines and cytokine receptors
Determined were: TNF, IL-6, sTNF-R55 and sTNF-R75 by means of ELISA's (see 2.5)

Acute phase proteins
Determined were: CRP, α1-antitrypsin, α1-acid glycoprotein, C3, C4, albumin and transferrin (see 2.2 and 2.4).

Results

Clinical features
After ILP all patients developed chills and fever which were treated with 1 g paracetamol rectally. Mean body temperature rose from 36.9±0.1 °C to 39.2±0.2 °C at 4 hours post-perfusion and decreased to 37.3±0.1°C at 1 day after ILP. At baseline mean arterial blood pressure (MAP) was 100±5 mmHg. In all patients a decrease in MAP was observed during approximately 2-4 hours after ILP to a minimum value of 86±6 mmHg. Hypotension could be corrected with fluid replacement, without the need for vasopressors.

Cytokine and cytokine receptor profiles
Serum TNF, IL-6, sTNF-R55 and sTNF-R75 concentrations are shown in Fig 1. TNF levels at baseline and on the days of pretreatment with r-HuIFNγ (days -2 and -1) were below detection limits. After the end of ILP, defined as the moment of reconnection of the limb to the systemic circulation, leakage of TNF resulted in detectable TNF levels at 1 min in all patients. Maximum TNF levels were measured at time points between 1 min and 30 min and ranged from 490 to 565,400 pg/ml ($p<0.05$). Thereafter, TNF concentrations rapidly decreased and were no more detectable at 24 h after ILP.

IL-6 levels were below detection limits at baseline and during pretreatment at days -2 and -1. In each patient the TNF peak after ILP was followed by a marked increase in IL-6 levels with maximum values of 7820±3398 pg/ml at 4 h post-perfusion ($p<0.05$). Subsequently, IL-6 concentrations decreased and were not different from pretreatment values after day 2.

STNF-R55 and sTNF-R75 concentrations were within the normal range at baseline and during pretreatment with r-HuIFNγ. Directly after ILP, sTNF-R55 and sTNF-R75 levels showed similar changes in time with a marked increase at 5 min to 4.66±1.28 ng/ml and 4.90±1.03 ng/ml respectively ($p<0.05$), reaching maximum concentrations at 4 h post-perfu-

sion (sTNF-R55: 5.34±1.19 ng/ml; sTNF-R75: 6.48±1.24 ng/ml, $p<0.05$). Subsequently, sTNF-R55 and sTNF-R-75 levels decreased gradually but remained elevated ($p<0.05$) compared to baseline values throughout the study period. Renal function of all patients was normal at baseline and was not affected by the ILP procedure (data not shown).

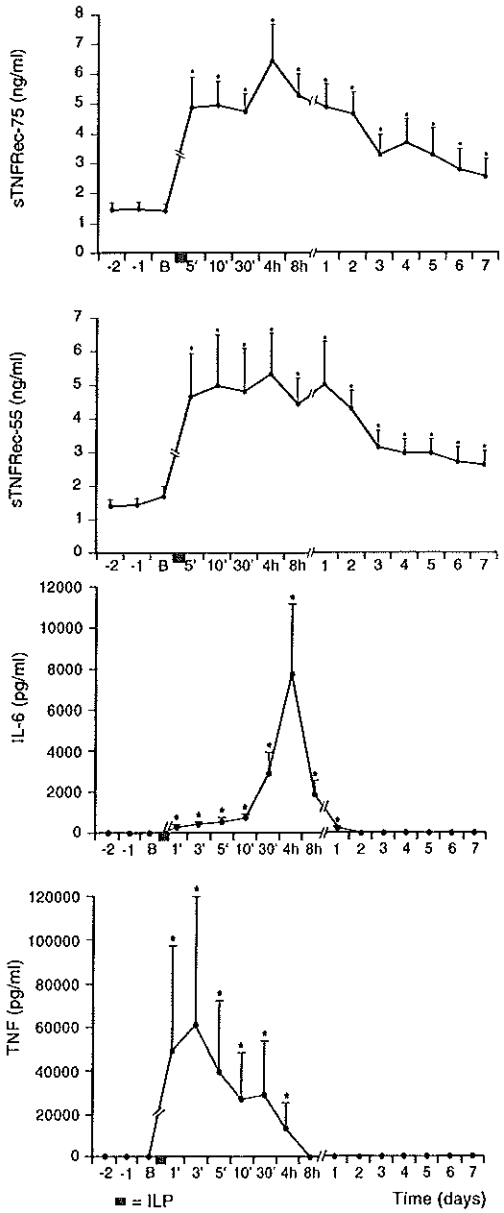

*Figure 1. Time courses of TNF, IL-6 and sTNF-R55 and -75 levels after ILP. Patients were pretreated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 1, 3, 5, 10, 30 minutes (') and at 4 and 8 hours (h). Subsequently, samples were obtained at 7 consecutive days after ILP. Data are expressed as mean ± SEM. *$p<0.05$ compared to baseline.*

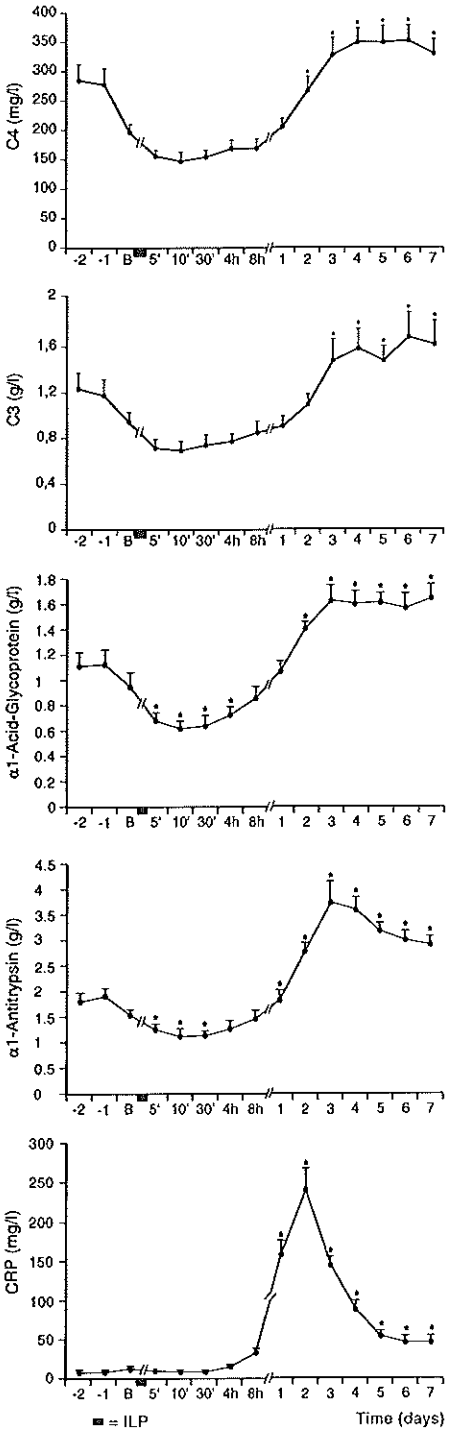

*Figure 2. Time course of the levels of the acute phase proteins CRP, α1-antitrypsin, α1-acid-glycoprotein, C3 and C4 after ILP. Patients were pre-treated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 5, 10, 30 minutes (') and at 4 and 8 hours (h). Subsequently, samples were obtained at 7 consecutive days after ILP. Data are expressed as mean ± SEM. *p<0.05 compared to baseline.*

Time course of positive acute phase proteins

The time courses of CRP, α1-antitrypsin, α1-acid-glycoprotein, C3 and C4 concentrations are presented in Fig. 2. Pretreatment values of APP were within the normal range, except for α1-acid-glycoprotein levels which were slightly elevated. CRP levels started to increase at 4 h after ILP and rose to maximum levels at day 2 (232±28 mg/l, $p<0.05$). Subsequently, CRP levels decreased, but remained elevated compared to baseline values until day 7 ($p<0.05$).

The levels of α1-antitrypsin, α1-acid-glycoprotein, C3 and C4 were slightly decreased at baseline (NS) compared to day -2. Directly after ILP, α1-antitrypsin, α1-acid-glycoprotein, C3 and C4 fell to 74±7 %, 70±6 %, 75±7 % and 75±6 % of baseline values respectively at 10 min ($p<0.05$). Subsequently, α1-antitrypsin and α1-acid-glycoprotein levels recovered at 4 h and started to rise at day 1 to maximum levels at day 3 (3.8±0.39 g/l and 1.66±0.12 g/l respectively, $p<0.05$) after which both remained elevated ($p<0.05$). C3 and C4 levels returned to baseline values at day 1 followed by an increase to maximum levels at day 6 (1.67±0.22 g/l and 353±26 mg/l respectively, $p<0.05$).

Time course of negative acute phase proteins

The time course of albumin and transferrin levels is shown in Fig. 3. At baseline, both albumin (-15±2 %, NS) and transferrin (-22±4%, $p<0.05$) levels were decreased compared to day -2. After ILP albumin levels fell from 36±4 to 22±2 g/l ($p<0.05$) and transferrin levels decreased from 2.1±0.2 to 1.3±0.1 g/l ($p<0.05$) at 10 min. In contrast to positive APP's, albumin and transferrin concentrations recovered only partially and remained at 72±6% to 77±10% of baseline values until day 2 ($p<0.05$). Subsequently, albumin and transferrin concentrations increased gradually, however, without reaching initial values.

Discussion

Treatment of cancer patients with regional ILP with high-dose r-HuTNF, r-HuIFNγ and melphalan was followed by induction of the acute phase response with associated changes in cytokine profiles and APP concentrations.

Cytokine network

We observed high systemic TNF levels directly after ILP, presumably due to resorption of residual TNF from the limb into the systemic circulation. It could be argued that endogenous TNF production, triggered by the surgical procedure itself, has contributed to circulating TNF levels. However, in early studies with ILP without r-HuTNF, systemic TNF levels were only

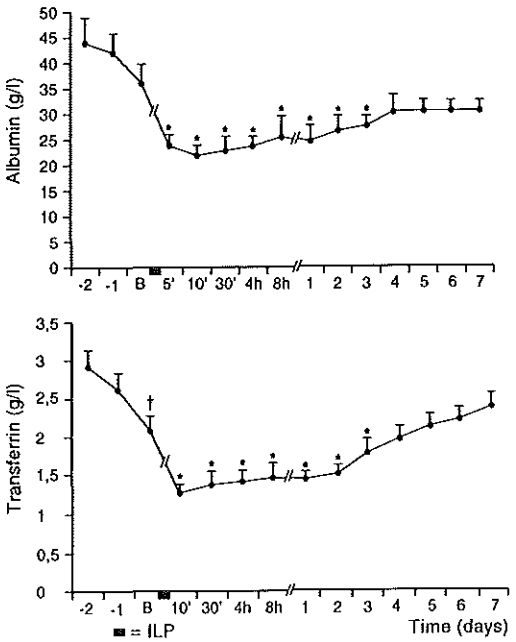

*Figure 3. Time course of the levels of the negative acute phase proteins transferrin and albumin after ILP. Patients were pretreated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 5, 10, 30 minutes (') and at 4 and 8 hours (h). Then, samples were obtained at 7 consecutive days after ILP. Data are expressed as mean ± SEM. *p<0.05 compared to baseline.*

minimally elevated, i.e. 0.01 to 0.1 % of TNF levels as measured after infusion of TNF (28,29). In addition, it has been shown previously that minor limb surgery under general anaesthesia is not accompanied by detectable TNF or IL-6 levels (30). Maximum TNF levels were higher than TNF levels reported in sepsis (31). These high TNF concentrations, though, were accompanied by only minor systemic toxicity, which is in accordance with previous studies on ILP with TNF (25,28). First, this might be related to a concomitant release of antiinflammatory substances, e.g. soluble TNF receptors (see below). Second, the mild clinical course might be explained by a rapid TNF clearance and the absence of a continuous stimulus for TNF production, either locally or systemically, as seen in sepsis.

The TNF peak was followed by the induction of IL-6. The early appearance of IL-6 at 3 min post-perfusion might be attributed to washout from the limb of locally produced IL-6 (29). The subsequent IL-6 peak at 4 h after the TNF peak is likely to represent systemic IL-6 induction. *In vitro*, TNF stimulates IL-6 gene expression by a variety of cell types including fibroblasts, macrophages and endothelial cells (11). *In vivo*, TNF administration results in a rapid induction of circulating IL-6 (9,10) which is confirmed by our results. IL-6, in turn, down-regulates TNF production (32), providing a negative feedback mechanism.

The inflammatory response is counteracted by several mechanisms including: (a) negative feedback loops via for instance IL-6 (32) and cortisol (see chapter 8); (b) production of antiinflammatory cytokines, e.g. IL-4 and IL-10 (33); (c) release of cytokine receptor antagonists, e.g for IL-1 (34), and soluble cytokine receptors, e.g. for TNF (24). The acute rise in TNF levels after ILP was followed by a rapid increase of sTNF-R55 and sTNF-R75 levels, suggesting that TNF is an important stimulus for sTNF-R release. Clearance of circulating sTNF-R's is thought to be mediated by the kidney and elevated sTNF-R levels have indeed been found in patients with renal failure (23). As renal function remained unaffected in our patients the rise in sTNF-R's is not likely to be explained by a decreased clearance. In contrast to TNF concentrations, sTNR-R levels decreased gradually and remained elevated during the study period. This is in accordance with kinetics of TNF and sTNF-R's observed in critically ill patients and in healthy volunteers challenged with endotoxin (20,24), indicating a sustained presence of sTNF-R's in response to an inflammatory stimulus. Both TNF-R55 and TNF-R75 are expressed by a variety of cell types and each receptor signals distinct TNF activities (16). *In vitro* studies have shown that neutrophils and monocytes are important cellular sources of sTNF-R's (35). Shedding of TNF-R's can be induced by various inflammatory stimuli, e.g. endotoxin, and cytokines like TNF and IL-10 (24,35).

An increased release of TNF-R's may attenuate TNF bioactivity. First, a decrease in membrane TNF-R number may concomitantly reduce cellular sensitivity for TNF. Second, sTNF-R's can bind circulating TNF, thereby limiting interaction of ligand with the cellular TNF-R. Indeed, in experimental animals with E.Coli-induced sepsis, administration of recombinant [r-] sTNF-R55 prevents the detection of circulating TNF and has beneficial effects on haemodynamic parameters (24). Reported sTNF-R levels in various clinical conditions are usually higher than TNF levels and the question arises whether these receptors bind all circulating TNF and can fully neutralize its bioactivity. The answer is presumably no. Van Zee et al. demonstrated that in endotoxaemic volunteers plasma sTNF-R levels are about ten fold higher compared to TNF levels (24). However, endogenous TNF-bioactivity of this plasma *in vitro* could significantly be diminished by co-incubation with r-sTNF-R55 or r-sTNF-R75. In addition, *in vitro* TNF-bioactivity of plasma samples containing r-HuTNF levels as observed in lethal sepsis could only partially be diminished by addition of supraphysiological amounts of r-sTNF-R55 or r-sTNF-R75 (24). Thus, a considerable proportion of total TNF is likely to circulate in its free form and biological responses to TNF may only partially be attenuated by sTNF-R's. In the present study only free TNF concentrations were determined. However, to assess the exact ratio between bound and unbound circulating TNF in various clinical conditions, future studies should focus on simultaneous measurements of free TNF and TNF complexed to sTNF-R's.

Acute phase response

Regulation of the hepatic APR is thought to be mediated by cytokines (1,2). Our data show the time dependency between TNF- and IL-6 profiles and the coordinate changes in APP levels. The levels of all plasma proteins examined were slightly decreased at baseline, presumably based on haemodilution, although an effect of IFNγ can not be excluded (36). The initial fall of APP directly after ILP, coinciding with TNF peak levels and a slight decrease in blood pressure, might be attributed to haemodilution and/or extravasation due to TNF-induced capillary leakage. Subsequently, positive APP profiles showed a sequential response with changes occurring at different rates and to different degrees. CRP, as representative of early APP's, started to rise 4h after the TNF peak when maximum IL-6 levels were found and reached peak levels at day 2. α1-Antitrypsin, α1-acid glycoprotein and complement factors C3 and C4, reflecting the second APP wave, increased at 24h after ILP to maximum levels at day 3 to day 6. In contrast, albumin and transferrin, known as negative APP, remained low until 2 days after ILP. It could be argued that cancer itself can induce an acute phase response. However, APP levels were normal before treatment, presumably since our patients have local tumors without systemic involvement.

The observed alterations in APP levels may reflect corresponding changes in hepatic APP synthesis, induced by the activated cytokine network. *In vitro*, cytokines modulate protein synthetic rate of APP as has been shown by studies with rat hepatocytes, human hepatoma cell lines and human hepatocytes. IL-6 appears to be the major regulator of APP synthesis, whereas cytokines like TNF and IL-1 may serve as accessory mediators (1,2,12-15). In human hepatocytes IL-6 stimulates the full spectrum of APP (type 1 and 2) including CRP, α1-antitrypsin, α1-acid glycoprotein and C3 and C4, and its effect is potentiated by glucocorticoids. TNF and IL-1, however, induce only a subset of APP (type 1) and to a lesser magnitude compared to IL-6 (13,14,37,38). Both IL-6, TNF and IL-1 inhibit the synthesis of albumin and transferrin (12,14). Thus, the observed changes in APP levels in this study may primarily be mediated by IL-6. *In vivo* studies with ^{125}I-IL-6 in rats have shown that the liver is indeed a target organ for IL-6 and that IL-6 induces hepatic IL-6 receptor expression (14). IL-6 can reach hepatocytes by an endocrine route, but also by a paracrine route via local IL-6 production by Kupffer cells. Additional mediators like TGF ß1 and IL-6 type cytokines, i.e. leukemia inhibitory factor, IL-11, oncostatin M and ciliary neurotrophic factor, also modulate APP synthesis *in vitro*, their role *in vivo*, however, has not been clarified yet (1,2). It must be emphasized that *in vivo* hepatocytes are exposed to a mixture of cytokines rather than to individual mediators. An interesting observation in this respect is that the induction of APP's is not impaired in IL-6 deficient mice, illustrating the redundant action of IL-6 type cytokines (39).

Cytokine-controlled APP synthesis is primarily regulated at transcriptional level (1,2,13,38). Binding of the cytokine to its receptor initiates a signal transduction pathway resulting in activation of transcription factors such as nuclear factor-IL-6 [NF-IL-6] and acute phase response factor which interact with the 5'untranslated regulatory region of APP genes (1,2,40-42). Cytokines are thought to regulate the synthesis of these transcription factors as well. For instance, IL-6 stimulates the synthesis of NF-IL-6 mRNA *in vitro* (41). Cytokines may also modulate APP synthesis at translational and post-translational level as has been shown for CRP (43,44). Therefore, regulation of APP synthesis is highly complex and includes cooperative action of multiple cytokines which modulate APP synthesis at different levels.

In conclusion, TNF administration in humans activates the cytokine network with induction of the acute phase response, presumably with an important regulatory role for IL-6. Simultaneously, counter-regulatory mechanisms, such as the release of soluble TNF receptors, are activated to control the inflammatory response.

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Chapter 7

REGULATION OF IRON METABOLISM IN THE ACUTE PHASE RESPONSE

Interferon γ and tumor necrosis factor α induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients

Introduction

The host response to infection, inflammation and malignancy includes a broad spectrum of haemodynamic, metabolic and immunological reactions, collectively known as the acute phase response (1,2). Cytokines like TNF, IL-1 and IL-6 have been implicated in the regulation of the acute phase response (2-5, chapter 6). In the acute phase response major changes occur in iron metabolism resulting in: (a) low serum iron levels; (b) normal to increased iron stores; (c) elevated serum ferritin concentrations; (d) decreased levels of serum transferrin and transferrin iron saturation; (e) modulation of transferrin glycosylation (6,7).

Conversion of the acute phase response to the chronic inflammatory state, i.e. in the course of chronic infectious, inflammatory and neoplastic disorders, is frequently accompanied by the development of anaemia, defined as anaemia of chronic disease [ACD] (8-10). ACD is characterized by hypoferraemia in the presence of adequate iron stores (6,8,9) and the induction of ACD is related to the release of proinflammatory cytokines (11,12). Pathogenetic factors in ACD include inhibition of erythroid proliferation by cytokines like TNF, IL-1 and IFNγ (13-15), an impaired erythropoietin response to the anaemia (16-18) and a decreased erythroblast iron availability (6,8,9).

Several mechanisms contribute to this impaired erythroblast iron availability. First, iron is redistributed towards storage sites, confirmed by an increase in stainable iron located in liver and MPS (19,20). This iron shift may result from an increased influx of iron into storage compartments (21,22). Concurrently, iron release by MPS and liver may be impaired, which is associated with an increased ferritin synthesis (23-26). It has been speculated that modulation of intracellular iron processing results in a diversion of labile iron into the enhanced apoferritin pool (25). The exact mechanism, however, has not been clarified yet.

Second, iron uptake by erythroblasts is reduced in ACD as has been shown by both *in vivo* and *in vitro* studies (27,28), which may rely on a decreased erythroid transferrin receptor [TfR] expression as has been shown in chapter 4. Serum TfR levels reflect tissue TfR expression and rate of erythropoiesis (29-32). In ACD, sTfR levels are relatively low (33, chapter 5) and may therefore represent an impaired erythroid growth and TfR turnover. Iron transport to erythroid precursors in ACD may further be modulated by alterations in transferrin glycosylation. In chapter 3 we found that the induction of ACD in RA is associated with a shift in the transferrin microheterogeneity pattern towards variants with highly branched glycan chains. It remains, however, speculative whether these changes in transferrin glycosylation significantly influence iron donation to target cells.

The alterations in iron metabolism in ACD may be mediated by cytokines. In rodents, both

TNF, IL-1 and IL-6 induce hypoferraemia *in vivo* (22,34,35). *In vitro*, these cytokines modulate iron handling as well as ferritin synthesis by rat hepatocytes, human hepatoma cell lines and macrophages (22,36-38). In addition, cytokines like IL-6 influence glysosylation patterns of various acute phase proteins *in vitro* and *in vivo* (39), and may thus regulate transferrin glycosylation as well in the acute phase response. In chapter 3 it was shown that in ACD in RA changes in iron metabolism are correlated with serum levels of TNF and IL-6. The effects of cytokines on iron metabolism in humans, however, are presently unknown. In chapter 6 we demonstrated that ILP with r-HuTNF in patients with melanoma or sarcoma is followed by a transient systemic TNF peak with subsequent induction of IL-6 and the acute phase response.

In this study, we examined the role of TNF in the regulation of iron metabolism in the acute phase response by serial measurements of iron status, sTfR levels and transferrin microheterogeneity patterns in cancer patients treated with ILP with r-HuTNF.

Patients and Methods

Patients

Twelve patients were studied with irresectable soft tissue sarcoma (n=8) or melanoma with multiple in transit melanoma metastases (n=4). Demographic data are described in chapter 6.

Isolated limb perfusion

The method of ILP is described in 2.10. In short, patients are pretreated daily with 0.2 mg r-HuIFNγ subcutaneously on the two days before ILP. The ILP procedure is carried out under general anaesthesia. After cannulation of the local vasculature, the limb is perfused via an extracorporeal circuit during 90 minutes with r-HuTNF (2 mg/arm or 4mg/leg) and 0.2 mg r-HuIFNγ. Melphalan (13 mg/l arm volume or 10 mg/leg volume) is administrated after 30 minutes. ILP is followed by drainage of the perfusate from the limb and a washout procedure. After the ILP procedure patients are extubated and observed at the intensive care unit for 24 hours.

Blood sampling

Before ILP samples were obtained on the two pretreatment days (day -2, day -1) and at baseline. After the end of the ILP procedure, defined as the moment of releasing the tourniquet with reconnection of the limb to the systemic circulation, serum was collected at 1, 3, 5, 10 and 30 minutes [min] and 4 and 8 hours [h]. Subsequently, serum was obtained on 7 consecutive days after ILP at 8.00 h.

Laboratory procedures

Cytokines

Determined were: TNF and IL-6 by means of ELISA's (see 2.5)

Haemoglobin, iron status and serum transferrin receptor

Determined were: Hb, serum iron, serum transferrin, serum ferritin and serum transferrin receptor concentrations (see 2.2, 2.3 and 2.9).

Transferrin microheterogeneity

Transferrin microheterogeneity was assessed by isoelectric focusing [IEF] using the Phast-System. This technique is described in detail in 2.7. Briefly, iron-saturated serum samples are applied to an immobiline gel with a pH gradient 5-6. The transferrin isotypes are subsequently separated, based on differences in sialic residues, by IEF. After immunoprecipitation and staining, the sialo-transferrin bands are quantitated densitometrically. Using IEF, 9 transferrin subfractions (0 to 8-sialotransferrin) can be identified. Analogous to the approach in chapter 3, the transferrin isotypes were divided into three subgroups: the low sialylated transferrin fractions (LSTf, the sum of 0, 1, 2 and 3-sialotransferrin), the 4-sialotransferrin fraction (4-STf, as this is the predominant fraction in normal serum) and the highly sialylated transferrin fractions (HSTf, the sum of 5, 6, 7 and 8-sialotransferrin). The distribution of these transferrin subgroups in healthy individuals is as follows: LSTf: 12 ± 2%; 4-STf: 64 ± 3 %; HSTf: 24 ± 2 %. Fig. 1 shows a transferrin microheterogeneity pattern as assessed by IEF and densitometry.

Results

Clinical features

Clinical characteristics are described in chapter 6 (results).

Cytokine profiles

The time course of serum TNF and IL-6 levels is shown in Fig 2. and is described in detail in chapter 6. In short, directly after ILP TNF levels rapidly increase to maximum concentrations at 3 min post-perfusion. Thereafter, TNF concentrations decreased and were no more detectable at 24 h after ILP. The TNF peak was followed in each patient by an increase in IL-6 levels with maximum concentrations at 4 h after ILP. Subsequently, IL-6 concentrations decreased and were not different from pretreatment values after day 2.

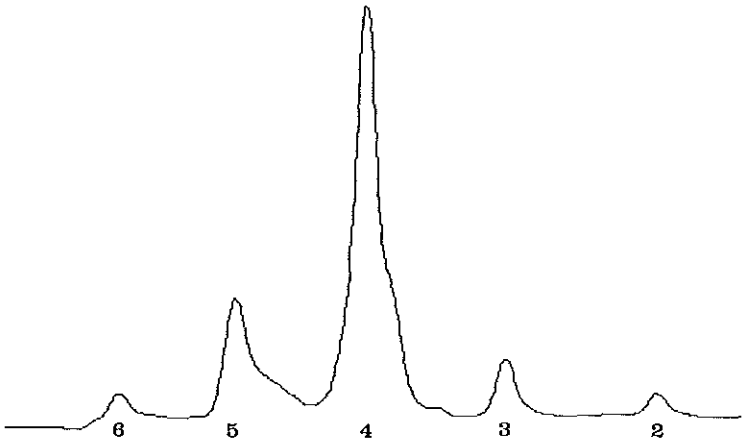

Figure 1. Transferrin microheterogeneity pattern as assessed by isoelectric focusing and subsequent densitometric scanning. The indices beneath the pattern indicate the number of sialic acids attached to the N-linked glycans of corresponding subfractions.

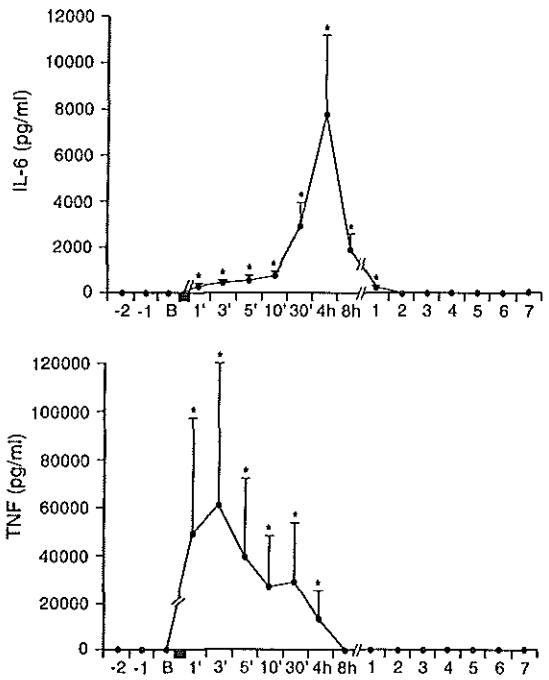

*Figure 2. Time courses of TNF and IL-6 levels after isolated limb perfusion (ILP). Patients were pretreated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 1, 3, 5, 10, 30 minutes (') and at 4 and 8 hours (h) and 7 consecutive days. Mean ± SEM. *p<0.05 compared to baseline.*

Iron status, serum transferrin receptor and haemoglobin

The time courses of serum transferrin, iron, ferritin, CRP and sTfR levels are shown in Fig. 3. Serum transferrin levels, which were normal at baseline, parallelled albumin levels with a decrease at baseline compared to day -2 (-22±4%, $p<0.05$) and a subsequent fall to 63±5% of baseline levels at 10 min post-perfusion ($p<0.05$). Transferrin levels then remained at 72±6% to 75±6% until day 2 ($p<0.05$) followed by a gradual recovery.

Serum ferritin levels were elevated at baseline compared to day -2 with a relative increase of 55±29% (NS). Directly after ILP at 10 and 30 min, when all APP except for CRP dropped (see chapter 6), ferritin levels rose further with a sharp increase at 8 h to 299±82 µmol/l ($p<0.05$) and parallelled CRP levels to peak values at day 2 (+173±65%, $p<0.05$), followed by a gradual decrease. Serum iron levels fell during the pretreatment period from 18.3±1.4 to 9.1±0.8 µmol/l at baseline ($p<0.05$). After ILP, serum iron levels remained unchanged at 10 and 30 min and showed a marked decrease at 8 h and day 1 to 3.1 µmol/l (-64±3%, $p<0.05$) and 3.3 µmol/l (-58±8%, $p<0.05$) respectively, occurring simultaneously with the rise in serum ferritin. Subsequently, serum iron levels returned to baseline values at day 2 and rose transiently over pre-TNF values at day 3 and 4 (NS).

Basal sTfR concentrations were within the normal range. During pretreatment with r-HuIFNγ, sTfR levels fell from 6.0±0.8 to 3.4±0.3 mg/l at baseline ($p<0.05$) followed by a further decrease after ILP to 3.0±0.7 mg/l at day 2. Then, sTfR levels increased, however, without reaching initial values. Mean Hb level before ILP was 8.5±0.2 mmol/l and fell to 5.3±0.3 mmol/l at 1 h post-perfusion which is attributed to haemodilution and blood loss due to the washout procedure of the limb. At day 1, day 2 and day 5 after ILP Hb levels increased to 6.7±0.2, 6.7±0.3 and 7.2±0.3 mmol/l respectively.

Transferrin microheterogeneity

Fig. 4a shows the time course of transferrin subfraction concentrations. Pretreatment levels were within the normal range. After ILP, both LSTf, 4-STf and HSTf decreased to minimal values at day 1 ($p<0.05$), in parallel with the decrease in total transferrin concentrations (see Fig. 3). During the subsequent recovery of transferrin, 4-STf and HSTf levels returned almost to initial values, whereas LSTf concentrations remained decreased compared to baseline levels ($p<0.05$). Fig. 4b shows the time course of transferrin subfraction percentages. Baseline values were within the normal range. No significant changes were observed in percentual distribution of transferrin subfractions during pretreatment with IFNγ and after TNF administration.

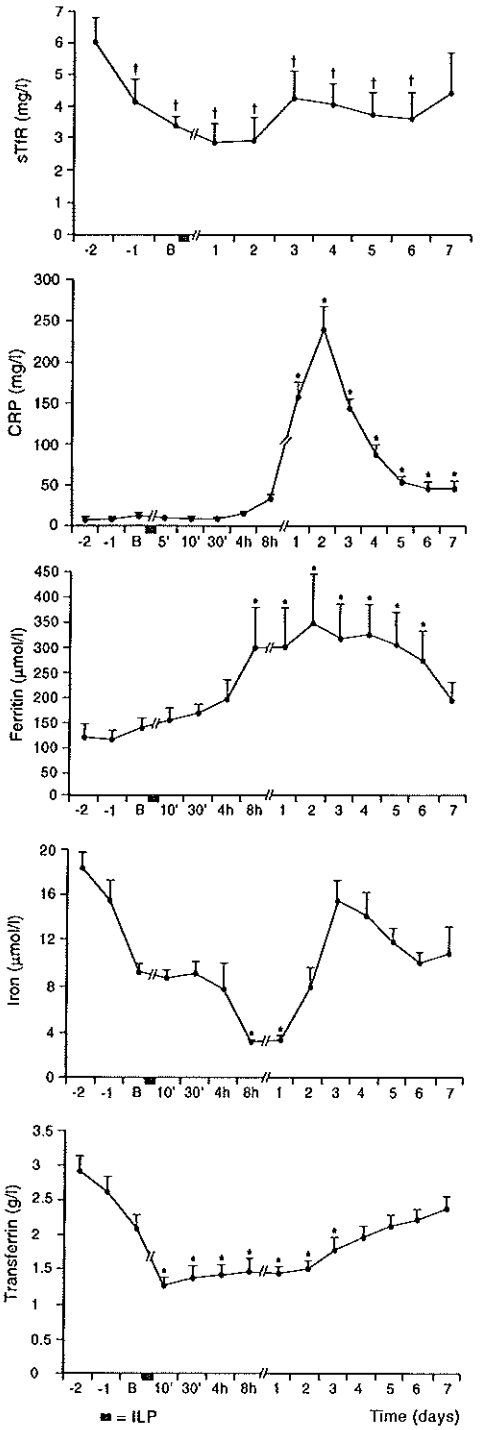

*Figure 3. Time courses of serum transferrin, iron, ferritin, CRP and transferrin receptor levels after isolated limb perfusion (ILP). Patients were pretreated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 10 and 30 minutes (') and at 4 and 8 hours (h) and 7 consecutive days. Mean ± SEM. *p<0.05 compared to baseline, †p<0.05 compared to day -2.*

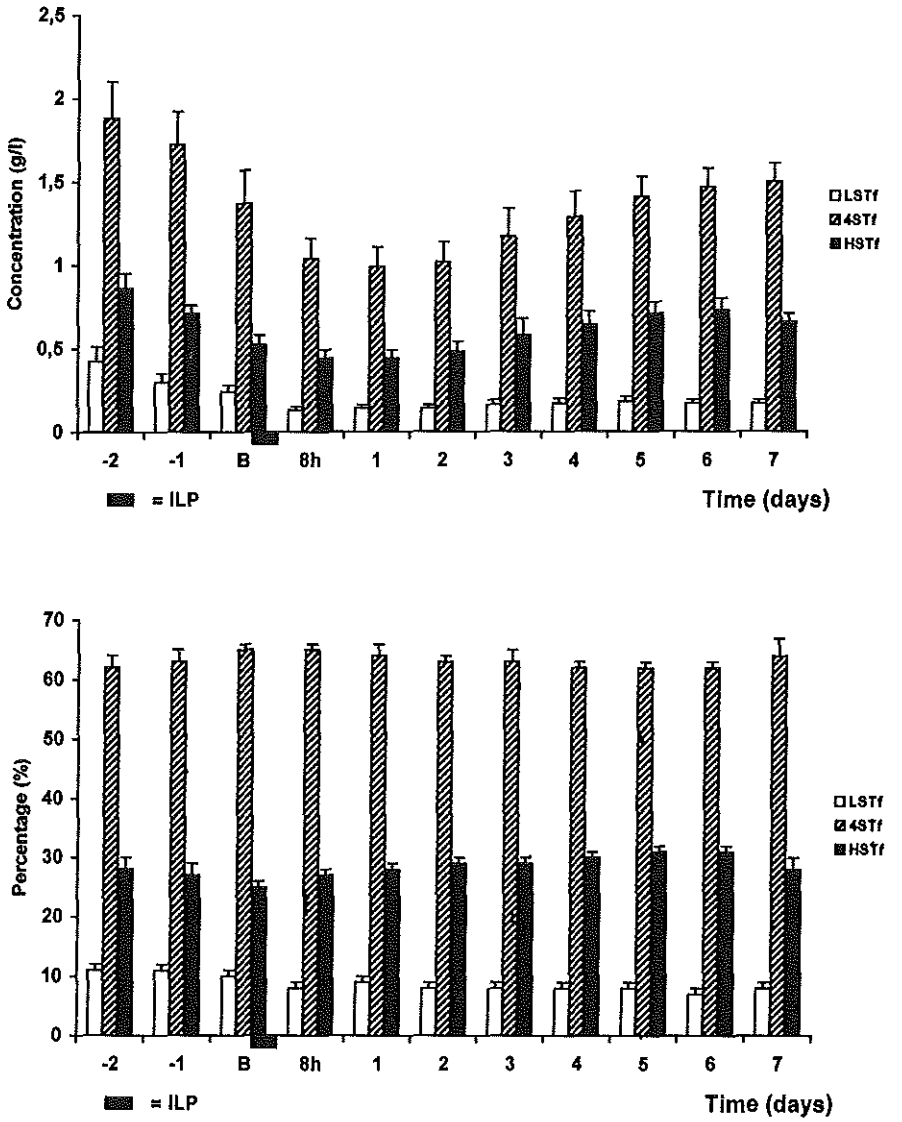

Figure 4a (top) and b (bottom). Time course of transferrin subfraction concentrations and percentages after isolated limb perfusion (ILP). LSTf, low sialylated transferrin fractions; 4-STf, 4-sialo transferrin; HSTf, highly sialylated transferrin fractions. Patients were pretreated with r-HuIFNγ at day -2 and -1. At the day of ILP measurements were performed at baseline (B) and after the end of ILP at 8 hours (h) and 7 consecutive days. Mean ± SEM.

Chapter 7

Discussion

The aim of this study was to examine the role of cytokines in the regulation of iron metabolism in the acute phase response. We therefore evaluated the effects of r-HuTNF administration, after r-HuIFNγ pretreatment, on iron status, sTfR levels and transferrin microheterogeneity in cancer patients. Our data show that IFNγ and TNF administration results in major changes in iron metabolism characterized by hypoferraemia, increased ferritin production and decreased circulating sTfR concentrations.

Induction of hypoferraemia

Hypoferraemia in inflammation and ACD is associated with a diversion of iron into liver and MPS, reflected by low serum iron and elevated serum ferritin levels (6). These alterations in iron metabolism, which are similar in disorders of various etiology, are related to macrophage activation (12), pointing to a role for the cytokine network as common denominator. In rodents, TNF, IL-1 and IL-6 induce a rapid decrease in serum iron levels, occurring within 3 to 6 hours (22,34,35). We showed that administration of TNF in humans results, directly or indirectly via induction of IL-6, in hypoferraemia associated with an increased ferritin production. This is in accordance with the study of Biesma et al. who found similar changes in post-operative iron metabolism, preceded by an increase in IL-6, in patients who underwent total hip replacement (40). IFNγ may modulate iron metabolism as well since pretreatment with r-HuIFNγ was accompanied by a substantial decrease in serum iron and an increase in ferritin levels. This is in line with the inverse relationship found in patients with ACD between serum iron and neopterin, which is produced by macrophages after stimulation with IFNγ (12). Before cytokine administration, none of the patients had inflammation-associated changes in iron metabolism or ACD. The observed changes in serum iron and iron binding proteins can therefore not be attributed to the underlying cancer perse. Although all patients received erythrocyte concentrate, this is unlikely to influence iron status in this short-term study, considering expected erythrocyte survival.

Several mechanisms may be operative in cytokine-mediated hypoferraemia. First, iron influx into storage compartments may be modulated. In rats, treatment with IL-6 shortens plasma half-life of ^{59}Fe-labelled transferrin, which is associated with a concomitant increase in liver ferritin content (22). *In vitro*, IL-6 stimulates receptor-mediated endocytosis of ^{125}I-labelled transferrin by rat hepatocytes (22). Similarly, single-cytokine stimulation experiments with the human hepatoma cell line HepG2 have shown that TNF, IL-1 and IL-6 induce the synthesis and expression of transferrin receptors with a concomitant increased uptake of ^{59}Fe-labelled

transferrin (36). The effects of cytokines on iron incorporation by cells of the MPS are less clear. Transferrin uptake by macrophages obtained from TNF-treated mice was increased compared to controls (38). In contrast, iron uptake by rat Kupffer cells and a human monocytic cell line was unaffected or even decreased after exposure to TNF, IL-1, IL-6 or IFNγ (22,37). Therefore, mobilization of transferrin-bound iron to storage sites in the acute phase response may predominantly be mediated by hepatic uptake. In the setting of chronic inflammation, cytokines like TNF and IL-1 may augment erythrophagocytosis by the MPS resulting in an increased clearance of nonsenescent erythrocytes and thus an enhanced influx of haemoglobin-bound iron into the MPS (28,41).

Second, tissue iron release may be affected by cytokines. The MPS plays an important role in iron recirculation, iron obtained from heme degradation is either donated to transferrin or incorporated into ferritin, depending on bone marrow iron demand. Ferrokinetic studies in humans have shown that in inflammatory conditions and ACD iron release by the MPS is impaired, which correlates negatively with serum ferritin (24). In rats with turpentine-induced inflammation an increase in ferritin synthesis precedes the fall in serum iron (25). It has thus been hypothesized that an impaired iron release is based on a primary induction of apoferritin with a concomitant shift of labile iron into ferritin stores (25). Our data support this concept as we show that in humans reciprocal changes in iron and ferritin levels occur simultaneously in the acute phase response.

Regulation of ferritin synthesis in the acute phase response

The synthesis of ferritin and the TfR is primarily regulated by the intracellular iron concentration at post-transcriptional level via interaction of iron regulatory proteins [IRP] with iron responsive elements [IRE] located in the 5' and 3' untranslated regions of mRNAs encoding for ferritin and TfR respectively (42). In cellular iron deficiency, but also in the presence of nitric oxyde [NO], IRP is converted to an active form which binds with high affinity to IRE. As a result ferritin translation is repressed, whereas TfR mRNA is stabilized. Conversely, when cellular iron is in excess this mechanism is reversed (42,43). It could thus be argued that the increased ferritin synthesis in the acute phase response results merely from an increased iron influx. However, cytokines may stimulate ferritin synthesis independent of intracellular iron, possibly at both translational and transcriptional level.

In vitro, TNF, IL-1, IL-6 and IFNγ induce ferritin synthesis by a variety of cell types including hepatoma cells, macrophages, fibroblasts and muscle cells (36,37,44-48). In HepG2 cells TNF, IL-1 and IL-6 enhance ferritin translation without an accompanying decrease in TfR synthesis which is unaffected or even increased (36,44). This indicates that cytokines may

stimulate ferritin translation via an IRP-IRE independent mechanism or by modulation of the IRP-IRE-pathway. Indeed, stimulation of human monocytes by IFNγ is accompanied by a transient early increase in IRP activity followed by a prolonged down-regulation of IRP activity, associated with an increased ferritin synthesis (48). This might be mediated via induction of NO, known to modulate IRP activity (43), since prevention of NO formation abolishes the effects of IFNγ on IRP activity (48).

Cytokines may also control ferritin synthesis at transcriptional level with a possible role for the transcription factor NFκB (46,47). Interestingly, in monocytic and mesenchymal cell lines TNF selectively increases ferritin heavy chain mRNA, independent of intracellular iron (37,46, 47). Since the rate of iron incorporation into heavy chain rich ferritin is greater than into the light chain rich variant (49), preferential synthesis of H-subunits may enable the cell to respond rapidly to an increased iron influx and may be part of the mechanism diverting labile iron into ferritin in the acute phase response. Thus, substantial evidence point to a primary stimulation of ferritin synthesis in inflammation and ACD by cytokines via a non-iron mediated mechanism and from *in vitro* and our *in vivo* observations it can be inferred that TNF, IFNγ and IL-6 may have an important regulatory role. Cytokine-controlled ferritin synthesis, however, is complex and multiple pathways are involved, presumably depending on cell type and cytokine.

Hypoferraemia and anaemia of chronic disease

The present data show that hypoferraemia is an early feature of inflammation, induced within 8 hours after initiation of the acute phase response. The hypoferraemic response is thought to be part of host defense mechanisms by withholding vital iron from pathogenic micro-organisms or malignant cells (50). In addition, lowering of intracellular labile iron in macrophages may modulate several immune effector functions (51).

In conditions in which the acute phase response is converted to the chronic inflammatory state, continuous impairment of iron transport to bone marrow contributes to the development of ACD. Iron handling by erythroblasts is thought to be modulated as well since iron uptake and TfR expression by erythroid precursors may be decreased in ACD (27,28,52, chapter 4). Serum TfR levels are related to erythroid TfR turnover and in ACD sTfR levels are indeed low in the face of an impaired erythroblast iron availability as has been shown in chapter 5. In rats, inflammation is accompanied by a decrease in sTfR levels (53). We observed a significant fall in sTfR levels after administration of r-HuIFNγ and subsequently r-HuTNF. Erythroid TfR expression is primarily determined by erythroid proliferation rate and cellular iron requirements. The decrease in sTfR levels, which coincided with hypoferraemia, may thus reflect an impaired erythroid growth and/or TfR expression. First, this might be induced by IFNγ and

TNF which have been implicated as mediators of ACD. *In vitro*, IFNγ and TNF inhibit erythroid colony formation in a synergistic mode (14), whereas *in vivo* chronic administration of IFNγ or TNF results in anaemia (54,55). Second, the APP's α1-antitrypsin and H-ferritin, which were elevated during the entire study period (see chapter 6), suppress erythroid growth *in vitro* (56,57) and may thus have affected sTfR levels as well. Whether these APP's play a pathophysiological role in ACD has yet to be established. Although most of the administrated melphalan is presumably removed by the washout procedure, it can not be excluded that melphalan, which has potential bone marrow depressive effects, has influenced sTfR levels.

Transferrin microheterogeneity

In ACD in RA a shift is observed in transferrin microheterogeneity towards variants with highly branched glycans, correlating with serum TNF and IL-6 levels as decribed in chapter 3. Since these cytokines modulate glycosylation patterns of various APP's (39), we speculated whether transferrin glycosylation would be influenced by TNF. After TNF administration total transferrin levels decreased, possibly in part due to a decreased synthesis, with a parallel reduction in all transferrin subfractions. In this phase no evidence was found for preferential synthesis of highly sialylated transferrins. This might be explained by the relatively short duration of the inflammatory stimulus, i.e. the transient increase in TNF. The interval between onset of the acute phase response and the appearance of changes in transferrin glycosylation is presently unknown. These alterations may thus occur only after a prolonged exposure of hepatocytes to cytokines like TNF and IL-6. Future studies should focus on this time dependency as well as on *in vitro* effects of cytokines on the glycosylation pattern of transferrin synthesized by primary human hepatocytes.

During the recovery of transferrin the low sialylated transferrins remained decreased, in contrast to 4-sialo transferrin and the higher sialylated variants, although this did not significantly alter transferrin subfraction distribution. This prolonged decrease in low sialylated transferrins may be attributed to an increased clearance by an upregulated asialoglycoprotein receptor system (58). Alternatively, fine tuning of transferrin glycosylation may be directed to the 4-sialo and higher sialylated transferrins. These possibilities need, however further investigation.

In conclusion, the cytokine network with TNF as important proximal mediator plays a central role in the modulation of iron metabolism in the acute phase response and ACD. It should be noted that other mediators may be involved as well and further study is needed to clarify the complex interplay of cytokines and their role in the pathogenesis of ACD.

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Chapter 8

ASPECTS OF THE ENDOCRINE RESPONSE TO ACUTE INFLAMMATION

Tumor necrosis factor α activates the cortisol axis and induces the euthyroid sick syndrome in cancer patients

Introduction

The systemic response to tissue injury induced by trauma, infection, inflammation or neoplasia involves a coordinated series of systemic physiological reactions (1,2). This so-called acute phase response is accompanied by adaptive changes in endocrine systems reflected in altered plasma concentrations of for instance cortisol, catecholamines, thyroid hormones, prolactin, growth hormone and insulin (3,4). Regulation of the acute phase response is thought to be mediated by the activated cytokine network (2). TNF and IL-1 are proinflammatory cytokines within this network and both induce the release of other mediators, e.g. IL-6 (5-7, see chapter 6). These cytokines have been postulated to modulate the function of various endocrine axes in the inflammatory state (3,4). Conversely, several hormones influence production and biological effects of cytokines and participate in the regulation of the acute phase response.

This is illustrated by the interactions between the immune system and the hypothalamic-pituitary-adrenal [HPA] axis in the acute phase response. Activation of the HPA-axis, resulting in increased circulating cortisol levels, plays a central role in the host response to inflammatory stress and is thought to be mediated, at least in part, by proinflammatory cytokines (8). TNF, IL-1 and IL-6 may stimulate the HPA-axis at multiple levels (8-11). Cortisol, in turn, has many anti-inflammatory properties such as down-regulation of cytokine production (8,12,13).

Another endocrine adaptive response in the inflammatory state involves the modulation of thyroid hormone metabolism referred to as the euthyroid sick syndrome. This syndrome is characterized by low plasma T3 concentrations and increased levels of rT3 (14,15). T4 levels may be within the normal range, but are decreased with increasing severity of the underlying disease (16). TSH concentrations are usually normal, although slightly elevated or reduced TSH levels can also be found (17,18). The alterations in thyroid hormones in acute and chronic inflammatory states are remarkably similar despite heterogeneity of the underlying etiology. It has therefore been suggested that final common pathways are involved in the induction of the euthyroid sick syndrome with a central role for the activated cytokine network (19,20).

TNF, IL-1 and IL-6 have been shown to modulate thyroid hormone metabolism *in vivo* (21-23) and *in vitro* (24-27). Administration of TNF and IL-6 to humans provided an interesting model to evaluate the induction of the euthyroid sick syndrome, which occurred within hours (21,23). Conversely, inflammatory stimuli induced a less severe euthyroid sick syndrome in IL-6 knock-out mice (28). Considering these short-term characteristics of the changes in thyroid hormone metabolism related to TNF and IL-6, it could be speculated whether the recovery of the cytokine-induced euthyroid sick syndrome also follows certain characteristics.

In chapter 6 and 7 it was shown that ILP with r-HuTNF in cancer patients can serve as a

model to study the TNF-induced acute phase response in detail with respect to the induction of cytokines, acute phase proteins and alterations in iron metabolism. The aim of this study is to examine the effects of TNF on cortisol and thyroid hormone metabolism in the acute inflammatory state. First, since changes in these endocrine responses may influence various metabolic alterations within the acute phase response, e.g. regulation of acute phase protein [APP] synthesis (2). Second, to obtain more insight in cytokine-mediated alterations in thyroid hormone metabolism with respect to the induction and recovery of the euthyroid sick syndrome. We therefore measured plasma cortisol and thyroid hormone concentrations in relation to TNF and IL-6 levels during seven consecutive days in six cancer patients treated with ILP with r-HuTNF.

Patients and Methods

Patients

Three male patients with irresectable soft tissue sarcoma and three female patients with melanoma with multiple in transit melanoma metastases were studied. Median age was 54 (range 45-83) years.

Isolated limb perfusion

The method of ILP is described in 2.10. Unlike the patients in chapter 6 and 7 this subset of patients was not treated with r-HuIFNγ (see 2.10). In short, the ILP procedure is carried out under general anaesthesia. After cannulation of the local vasculature, the limb is perfused via an extracorporeal circuit during 90 minutes with r-HuTNF (2 mg/arm or 4mg/leg), melphalan (13 mg/l arm volume or 10 mg/l leg volume) is administrated after 30 minutes. ILP is followed by drainage of the perfusate from the limb and a washout procedure. After the ILP procedure patients are extubated and observed at the intensive care unit for 24 hours.

Blood sampling

Baseline samples were collected 24 hours [h] before ILP. After the end of the perfusion, defined as the moment of reconnection of the limb to the systemic circulation by releasing the tourniquet, plasma was collected at 5, 10, 30 minutes [min] and 4 h and subsequently at each of the seven consecutive days at 08.00 h.

Laboratory procedures

Cytokines

Determined were: TNF and IL-6 by means of ELISA's (see 2.5)

Hormones

Determined were: plasma cortisol, T4, T3, rT3, FT4, T3 uptake, TSH and TBG (see 2.6).

Results

Clinical features

All patients developed chills and fever after ILP, which were treated with 1 g paracetamol rectally. Mean body temperature rose from 37.0 ± 0.1 °C before ILP to 39.0 ± 0.3 °C at 4 hours post-perfusion and decreased subsequently to 37.2 ± 0.1°C at 1 day after ILP. At baseline, mean arterial blood pressure [MAP] was 98 ± 5 mmHg and during approximately 2-4 hours after ILP a slight decrease in MAP was observed to a minimum value of 90 ± 3 mmHg. There was no need for treatment with vasopressors.

Cytokines and cortisol

The changes in time of plasma TNF, IL-6 and cortisol concentrations are shown in Fig. 1. Baseline levels of TNF and IL-6 before ILP were below detection limits in all patients. After the end of ILP, defined as the moment of reconnection of the limb to the systemic circulation, TNF appeared into the circulation and rose to maximum values at 10 min (19,980 ± 4,669 pg/ml). Subsequently, TNF levels rapidly decreased and at 24 h TNF was not detectable in any of the patients. IL-6 levels started to rise at 5 min after ILP and reached maximal concentrations at 4 h (2,713 ± 715 pg/ml, $p<0.05$). Thereafter, IL-6 decreased and at day 2 IL-6 values were not different from pretreatment values. Baseline cortisol concentrations were within the normal range. The time course of cortisol parallelled that of IL-6: a rapid increase at 5 min (0.86 ± 0.15 μmol/l) followed by peak levels at 4 h post-perfusion (1.41 ± 0.23 vs. 0.52 ± 0.08 μmol/l at baseline, $p<0.05$). Cortisol levels were not different from pretreatment values from day 1 to day 7.

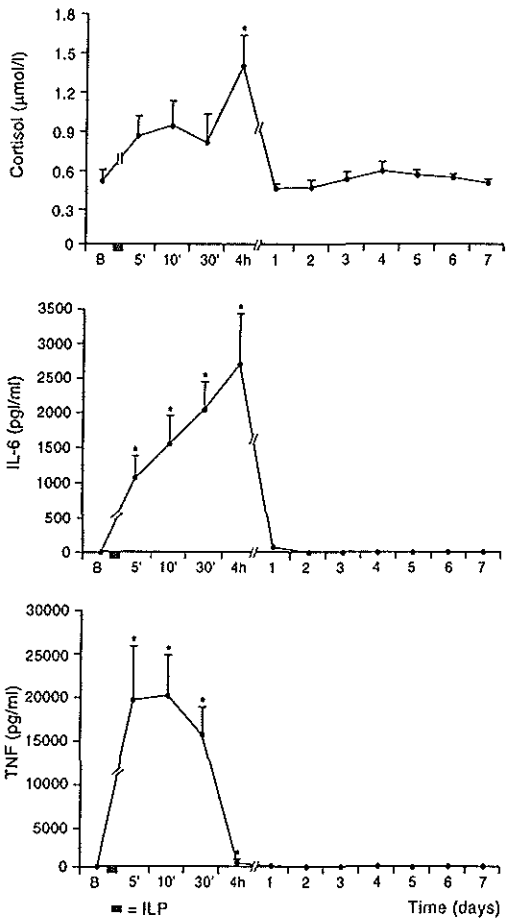

Figure 1. Time courses of plasma TNF, IL-6 and cortisol concentrations after isolated limb perfusion [ILP] with r-HuTNF and melphalan. Measurements were performed at baseline[B] and after the end of ILP at 5, 10, 30 minutes ['] and at 4 hours [h]. Subsequently, samples were obtained at 7 consecutive days after ILP. Data are expressed as mean ± SEM.
**p<0.05 compared to baseline.*

Thyroid hormones, TBG and TSH

Pretreatment levels of thyroid hormones, TBG and TSH were all within the normal range and are shown in Table 1. There were no differences in the responses of thyroid hormones between the patients with sarcoma versus those with melanoma. The time courses of thyroid hormones, TBG and TSH are presented in Fig. 2. ILP was followed by a rapid fall in TBG levels within 5 min post-perfusion (188 ± 20 vs. 337 ± 38 nmol/l at baseline, $p<0.05$). TBG concentrations remained low until day 1 and were not different from pretreatment values from day 2 to day 7. T4 levels paralleled TBG concentrations reflected in a decrease at 5 min (71 ± 10 vs 103 ± 12 nmol/l at baseline, $p<0.05$), low values at day 1 and subsequent recovery at day 2. Moreover, from day 5 to day 7 an increase of T4 levels exceeding basal levels was observed ($p<0.05$).

Table 1. Plasma concentrations of thyroid hormones, TBG and TSH at baseline in 6 cancer patients before isolated limb perfusion with rTNF and melphalan.

Subject no.	TBG nmol/l	T4 nmol/l	FT4 pmol/l	T3 - Uptake	T3 nmol/l	rT3 nmol/l	TSH mU/l
1	340	80	11.9	0.99	1.95	0.22	1.30
2	390	135	17.9	1.04	2.00	0.30	1.60
3	410	135	14.9	0.96	2.20	0.24	1.60
4	430	115	16.7	1.01	1.55	0.55	0.71
5	240	75	13.9	1.11	1.45	0.44	0.89
6	210	75	20.5	1.34	1.45	0.24	0.52
Mean	337	103	16.0	1.08	1.77	0.33	1.10
S.E.M.	38	12	1.2	0.06	0.13	0.05	0.19
Reference values	200-650	70-150	10-23	0.8-1.1	1.3-2.7	0.11-0.44	0.4-4

In contrast to total T4 concentrations, FT4 levels showed a marked increase from 16.0 ± 1.2 at baseline to 56.9 ± 9.6 pmol/l at 5 min post-perfusion ($p<0.05$). After 10 min FT4 levels gradually decreased and were not different from pretreatment values from day 1 to day 7. Similarly, the pattern of T3-resin uptake was characterized by a transient rise to maximum levels at 5 min ($p<0.05$). From day 1 to day 7 T3-resin uptake values were not different from pretreatment values.

After ILP a decrease was observed in both T3 (from 1.77 ± 0.13 to 0.70 ± 0.07 nmol/l, $p<0.05$) and rT3 levels (from 0.33 ± 0.05 to 0.10 ± 0.03 nmol/l, $p<0.05$) at 5 min post-perfusion. Subsequently, T3 concentrations remained low compared to pretreatment values until day 1, whereas rT3 increased over pretreatment values to maximum concentrations at day 1 (0.56 ± 0.12 nmol/l, $p<0.05$). Subsequently, T3 and rT3 levels recovered gradually, both returning to initial values at day 4.

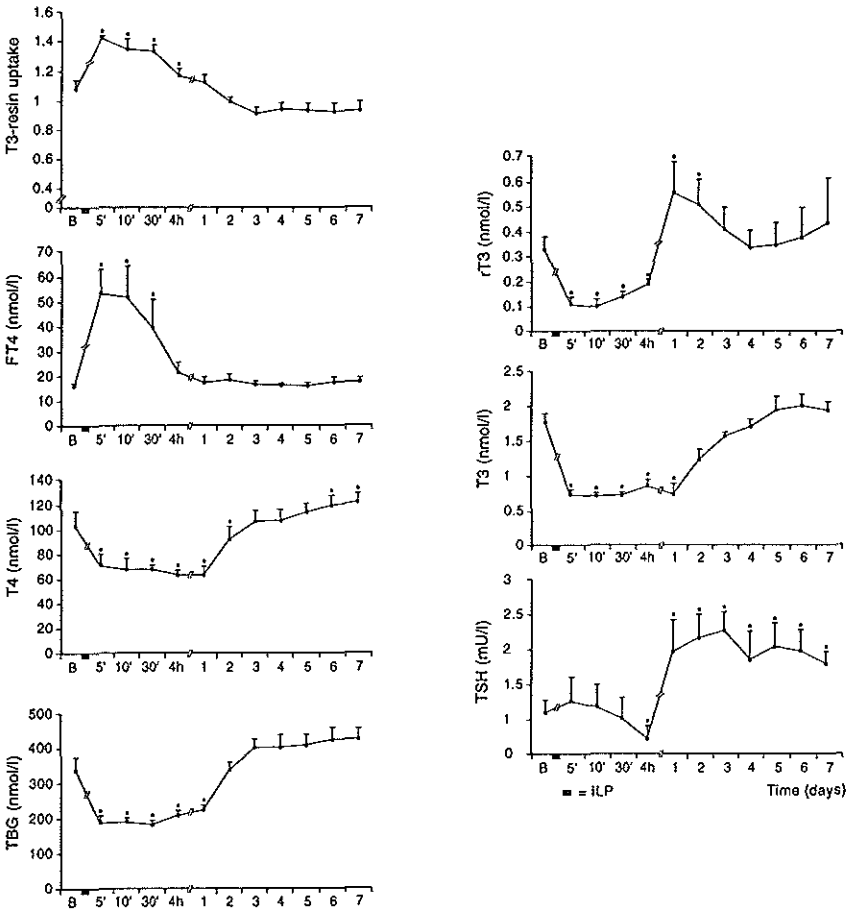

*Figure 2. Time courses of plasma TBG, T4, FT4, T3-resin uptake, TSH, T3 and rT3 concentrations after isolated limb perfusion [ILP] with r-HuTNF and melphalan. Measurements were performed at baseline [B] and after the end of ILP at 5, 10, 30 minutes ['] and at 4 hours [h]. Subsequently, samples were obtained at 7 consecutive days after ILP. Data are expressed as mean ± SEM. *p<0.05 compared to baseline.*

The time course of TSH showed an initial fall after ILP with minimum levels at 4 h (from 1.10 ± 0.19 to 0.72 ± 0.19 mU/l, $p<0.05$). Subsequently, a marked increase over pretreatment levels was observed at day 1 (1.99 ± 0.46 mU/l, $p<0.05$), preceding recovery of T4 and T3 concentrations in all subjects. TSH values remained elevated compared to pretreatment levels from day 1 to day 7 post-perfusion (relative increase from +94 ± 43 % to +155 ± 66 %, $p<0.05$).

Discussion

The aim of this study was to examine the effects of ILP with high dose r-HuTNF and melphalan on cortisol and thyroid hormone metabolism in cancer patients. Leakage of residual TNF into the systemic circulation was associated with the induction of IL-6 and a cortisol response and with major changes in plasma thyroid hormone concentrations compatible with the euthyroid sick syndrome.

Cytokine network and cortisol response

After ILP, resorption of residual TNF resulted in high systemic TNF concentrations with a subsequent induction of IL-6, similar to our findings in chapter 6 in which this is described in detail. This sequential increase in systemic TNF and IL-6 concentrations was followed by a rapid increase in circulating cortisol levels, indicating activation of the HPA-axis. This is in accordance with previous studies demonstrating stimulation of the HPA-axis after administration of proinflammatory cytokines (9-11). Infusion of TNF (50 $\mu g/m^2$) in healthy volunteers induced an increase in cortisol with maximum levels after 3 hours, preceded by a rise in ACTH (9). Similarly, IL-6 administration (30 $\mu g/kg$) in cancer patients was followed by a sequential increase in ACTH and cortisol concentrations (11).

There is substantial evidence that cytokines like TNF, IL-1 and IL-6 can independently stimulate the HPA-axis at all three levels and that, in combination, these mediators act in a synergistic mode (3,8-11,29-33). Early activation of the HPA-axis in the acute phase response is thought to be mediated at hypothalamic level since immunoneutralization of CRH blocks the acute stimulatory effects on ACTH and cortisol secretion induced by TNF and IL-1 (10,29). Apart from this short-term effect, TNF, IL-1 and IL-6 exert late stimulatory effects on ACTH and cortisol release by pituitary and adrenal glands respectively (31-33). For instance, cytokine-mediated cortisol secretion by the isolated adrenal cortex or in hypophysectomized animals occurs only after several hours (33). We observed a rapid recovery of cortisol levels at 24 h after a short-term inflammatory stimulus, i.e. a transient increase in systemic TNF concentrations. Apparently, prolonged activation of the HPA-axis requires a repetitive or continuous exposure to proinflammatory cytokines.

Activation of the HPA-axis plays an essential role in the host response to inflammation as the resultant increase in glucocorticoid levels has profound metabolic and immunomodulatory effects. Glucocorticoids have important antiinflammatory properties such as suppression of Th1 cell and macrophage activity with a concomitant down-regulation of cytokine production (8). Indeed, glucocorticoids strongly inhibit gene expression and secretion of cytokines like

TNF, IL-1 and IL-6 in different tissues (12,13,33). Thus, cytokine-mediated triggering of the HPA-axis provides a negative feedback loop to control the inflammatory response, next to counterregulatory mechanisms like release of antiinflammatory cytokines and soluble cytokine receptors (see chapter 6). On the other hand, glucocorticoids have been shown to synergize with cytokines like IL-6 in the induction of most hepatic APP's. Therefore, considering the time course of cortisol after ILP in this study, elevated cortisol levels may have potentiated the effects of TNF and IL-6 on APP production as observed in chapter 6. The effects of cortisol on iron metabolism in the acute phase response are presently unknown. In view of its inhibitory effects on macrophage function, it might be speculated that cortisol counteracts the effects of cytokines on iron handling by macrophages. This needs, however, further investigation.

Alterations in thyroid hormone metabolism: induction of the euthyroid sick syndrome

Following ILP, TBG levels promptly decreased associated with an acute fall in T4, T3 and rT3 concentrations. In chapter 6 it was shown that directly after ILP, most APP's decrease to approximately 60 % of initial values, presumably due to capillary leakage and haemodilution. Positive APP's like α1-antitrypsin recover within 4 h after ILP, followed by a subsequent increase. In contrast, negative APP's such as albumin remain low until day 2, which may result from inhibition of protein synthesis. In this respect TBG seems to behave like a negative APP. IL-6, a major regulator of APP gene expression, inhibits TBG synthesis by a human hepatoma cell line *in vitro* (34). Thus, the initial fall in TBG levels is likely to be caused by capillary leakage and haemodilution, whereas inhibition of TBG synthesis, e.g. by IL-6, may have contributed to the prolonged decrease in TBG levels.

Plasma T4 levels decreased acutely after TNF infusion which is predominantly related to the fall in thyroxine binding proteins like TBG. Whether an impaired thyroidal T4 release may be involved as well remains speculative. *In vitro*, TNF and IL-6 have inhibitory effects on TSH-stimulated thyroid cell functions like ^{125}I-incorporation, synthesis of thyroglobulin and thyroid peroxidase as well as thyroid hormone release (24-27). Moreover, TSH levels decreased which may have contributed to the prolonged decrease in T4 concentrations through a reduced stimulation of thyroid function. FT4 concentrations, in contrast, showed a dramatic increase directly after ILP which might be explained, at least in part, by effects of heparin. Heparin administration increases FT4 levels through an *in vitro* artifact caused by lipoprotein lipase-mediated generation of free fatty acids [FFA] (35). Alternatively, mediators like TNF, IL-6 and cortisol may affect T4 binding to TBG, e.g. by induction of FFA release *in vivo*. For instance, van der Poll et al. found a transient increase in FT4 levels, though within the normal range, and FFA concentrations after TNF-infusion in healthy volunteers who did not receive heparin (21).

TNF infusion was followed by the induction of the euthyroid sick syndrome as reflected in low T4 and T3 levels and elevated rT3 levels at 24 h post-perfusion. Kinetic studies in patients with the euthyroid sick syndrome have shown that low T3 levels are primarily caused by decreased peripheral conversion of T4 to T3 since thyroidal T3 production and T3 clearance are unaffected (36,37). Conversely, rT3 production is normal and elevated rT3 levels are caused by a decreased clearance (38,39). Several mechanisms may underly these reciprocal changes in T3 and rT3 levels with a possible role for cytokines. First, this involves an impaired hepatic type I 5'-deiodinase (5'D-I) activity (40). In rats TNF inhibits hepatic 5'D-I activity after one day of treatment (41). The effects of cytokines on human 5'D-I expression, however, are presently unknown. Second, hepatic T4 and rT3 uptake may be inhibited in nonthyroidal illness by factors such as FFA and bilirubin (42,43). Cytokines may also modulate hepatic thyroid hormone processing, this needs, however, further investigation.

The regulation of TSH secretion is altered in the euthyroid sick syndrome (14). TSH values are usually normal in nonthyroidal illness and seem to be inappropriately low in the face of reduced T3 and T4 levels. The initial fall in TSH levels in our study may be explained by the preceding rise in both FT4 and cortisol concentrations, known to suppress TSH secretion (14). In addition, TNF and/or IL-6 may have decreased TSH levels (21,23). For instance, in rats TNF induces a decrease in both hypothalamic TRH content and pituitary TSH expression (41). Thus, TNF and IL-6 may alter pituitary-thyroid relationships in the euthyroid sick syndrome, resulting in a blunted TSH responsiveness.

Apart from cytokines, the cortisol response may have affected thyroid hormone metabolism, e.g. by inhibition of T4-T3 conversion and suppressive effects on TSH secretion (14). It can not be excluded that the use of melphalan or anaesthetics has influenced our results. Unfortunately, there are no data available on the effects of these agents on thyroid hormone metabolism. The three men with sarcoma and the three women with melanoma included in our study all received the same treatment. It is unlikely that this difference in sex and tumor affected our conclusions as the changes in thyroid hormone levels were not different between both groups.

Recovery of the euthyroid sick syndrome

There are only few data available on the recovery of the euthyroid sick syndrome (44). We show that the initial phase of recovery from the acute euthyroid sick syndrome following TNF infusion occurs rapidly, i.e. within 1 day, reflected in increased TSH values. In all patients the recovery of T3 and T4 to pretreatment levels was preceded by a persistent and considerable rise in TSH values. This temporal relationship may imply that an increase in TSH is a prerequisite for recovery of thyroid hormone concentrations in nonthyroidal illness. This is in agree-

ment with the study of Hamblin et al. who demonstrated a concordance between a transient TSH rise and return of T4 to normal levels in critically ill patients (44). In contrast to their study, we observed that the recovery phase is associated with a rise in T4 levels exceeding pretreatment values which may be facilitated by the persisting overshoot of TSH. This overshoot of TSH and T4 concentrations occurred in the presence of FT4 and T3 values which were within the range of pre-TNF values. Thus, feed-back relationships within the hypothalamus-pituitary-thyroid-axis are altered during recovery of the euthyroid sick syndrome compared to both the induction phase of the euthyroid sick syndrome and to pretreatment relationships.

Functional aspects of the euthyroid sick syndrome

The euthyroid sick syndrome has been postulated to serve as an adaptive mechanism during illness to prevent excessive catabolism (45). The low T3 state would then reduce metabolic demands in nonessential tissues in order to conserve protein. However, the exact metabolic consequences of the decrease in T3 levels in nonthyroidal illness are still unclear and the question whether patients with the euthyroid sick syndrome are indeed biochemically euthyroid or rather hypothyroid has not been answered yet.

Chronic inflammation is frequently accompanied by the euthyroid sick syndrome and the anaemia of chronic disease [ACD]. It is not known, however, whether the euthyroid sick syndrome and ACD are clinically associated nor whether a pathogenetic interrelationship exists between both phenomena. Thyroid hormone may influence erythropoiesis in several ways. First, thyroid hormone exerts stimulatory effects on erythropoietin [EPO] production, both directly and indirectly via increased oxygen requirements due to an elevated metabolic rate (46,47). Second, thyroid hormone may directly enhance proliferation of erythroid precursors via a non-EPO related mechanism (48). Not surprisingly, anaemia, characterized by erythroid hypoplasia, is a common finding in hypothyroidism (49). Conversely, hyperthyroidism is frequently associated with an increased red cell mass (50). Although highly speculative, it can be hypothesized that decreased thyroid hormone concentrations in the chronic inflammatory state contribute to the development of the hypoproliferative erythropoiesis and impaired EPO response in ACD. This proposal needs, however, further investigation.

In conclusion, activation of the HPA-axis and induction of the euthyroid sick syndrome are part of the endocrine response to inflammation and are, at least in part, mediated by cytokines like TNF and IL-6. The cortisol response may potentiate cytokine-mediated APP synthesis but also down-regulates production of proinflammatory cytokines. The metabolic consequences of the euthyroid sick syndrome have not been elucidated yet. Recovery of the acute euthyroid

sick syndrome starts early and is associated with an overshoot of TSH and T4 but not of T3 and FT4 concentrations. These data illustrate that recovery from nonthyroidal illness, such as induced by TNF, has typical features with respect to thyroid hormone metabolism. The euthyroid sick syndrome should not only be interpreted in relation to the presence of nonthyroidal diseases, but also in relation to recovery of these diseases.

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Chapter 9

GENERAL DISCUSSION

9.1 Introduction

The host response to tissue injury involves a coordinated sequence of physiological reactions collectively known as the acute phase response. Challenges which commonly elicit the acute phase response include trauma, major surgery, infectious, inflammatory and malignant disorders. The acute phase response is viewed as an important adaptive and defense mechanism in order to maintain or restore homeostasis. The activated cytokine network, interacting with neuroendocrine systems, is presumed to play a major role in the the initiation and coordination of the acute phase response. The acute phase response may be transient with subsequent recovery, or convert to chronic inflammation (1-3).

Conversion of the acute phase response to the chronic inflammatory state is frequently accompanied by the development of ACD (4-6). ACD is a common cause of anaemia and is characterized by a hypoproliferative erythropoiesis. The pathophysiological basis of ACD is formed by the continuing inflammatory response. A typical feature of disorders accompanied by ACD is the production of proinflammatory cytokines. All processes thought to be involved in the development of ACD may be attributed to effects of these cytokines, including shortened erythrocyte survival, impaired erythropoiesis, disturbance of iron metabolism and a blunted EPO response to the anaemia (7). Nevertheless, many postulated actions of cytokines in the pathogenesis of ACD have yet to be established.

The aim of this thesis was to obtain more insight in the regulation of the acute phase response and the pathogenesis of ACD with respect to the role of cytokines, endocrine systems and iron metabolism. In part I, studies on iron metabolism in ACD were performed in an *in vivo* model of the chronic inflammatory state in patients with RA. In part II, TNF administration to cancer patients was used as a model to study various aspects of acute inflammation and systemic effects of TNF. In this chapter the obtained results are discussed and an effort is made to integrate all aspects studied into a broader concept. Considering acute and chronic inflammation as a continuum, observations made in an acute inflammatory model may also apply to the chronic inflammatory state and vice versa. Such interpretations should be made with caution, though, since various characteristics of the inflammatory response, e.g. patterns of expressed cytokines, endocrine responses etc., may differ in the acute versus the chronic stage.

9.2 Cytokine network and acute phase response

TNF is a proinflammatory cytokine and exerts a broad spectrum of biological activites. TNF has documented beneficial effects in host resistance to various microbial challenges, but may

also be a potential harmful mediator as illustrated by its pivotal role in the development of lethal septic shock (8). In addition, as suggested by its name, TNF is able to induce tumor cell necrosis and may therefore serve as an anti-cancer agent. Indeed, application of r-HuTNF in the treatment of patients with locally advanced extremity sarcomas and melanomas with ILP results in high response rates (9). In cancer patients treated with ILP with r-HuTNF we observed a transient increase in systemic plasma TNF levels after the procedure. This increase in TNF levels was followed by the induction of IL-6 and APP's, illustrating that TNF is a proximal mediator within the cytokine network with an important role in the initiation of the acute phase response (chapter 6). It must be stated that ILP with r-HuTNF as model to examine the acute phase response may have some limitations. First, the studies are performed in patients with a malignant disorder which itself may elicit an acute phase response. However, before TNF perfusion concentrations of cytokines and APP's were within the normal range, presumably as these patients have local tumors without widespread metastatic disease. Second, it can not be excluded that the infusion of melphalan and the use of co-medication has influenced the obtained results. However, considering potential side effects it is not attractive to administrate TNF to healthy volunteers and ILP with r-HuTNF in cancer patients can therefore serve as an alternative model to study systemic effects of TNF.

Despite high plasma TNF concentrations, which were even higher compared to TNF levels in sepsis, systemic toxicity after ILP appeared to be limited. Apart from the absence of a persistent inflammatory stimulus with continuing TNF production, this might be based on concomitant activation of antiinflammatory mechanisms (Table 1):

(a) The rise in TNF levels was paralled by a rapid increase in sTNF-R concentrations, which were, in contrast to TNF levels, not only elevated in the induction phase but also during the recovery phase of the acute phase response. In view of the counteracting effects of sTNF-R's on TNF bioactivity *in vitro* and *in vivo* (10), the prolonged appearance of sTNF-R's in the acute phase response may point to a functional role for sTNF-R's in protecting the host to excessive TNF activity. Increased sTNF-R levels have also been observed in critically ill patients and in chronic inflammatory disorders such as RA and systemic lupus erythematosus [SLE] (10,11). In RA, elevated TNF and sTNF-R levels have been found both in serum and in synovial fluid, correlating with disease activity (11). Whether therapeutic application of sTNF-R's is useful in conditions associated with disproportional TNF production is under current investigation.

(b) The TNF- and/or IL-6 mediated cortisol response after ILP, observed in chapter 8, is likely to down-regulate endogenous TNF and IL-6 production and their biological effects.

(c) Other antiinflammatory factors may have attenuated TNF activity such as the inhibitory cytokines IL-4 and IL-10. We did not assess the IL-1 system, i.e. IL-1β and its counterparts sIL-1-R's and IL-1RA after TNF-infusion. Since TNF acts synergistically with IL-1 and stimulates its production, it would be interesting to examine IL-1β, sIL-1-R's and IL-1RA levels, in addition to IL-4 and IL-10 levels, after TNF administration in future studies.

Table 1. Antiinflammatory mechanisms in the acute phase response.

* Production of antiinflammatory cytokines (e.g. IL-4, IL-10)
* Release of soluble cytokine receptors (e.g. sTNF-R, sIL-1-R)
* Release of cytokine receptorantagonists (e.g. IL-1RA)
* Activation of the hypothalamic-pituitary-adrenal axis

One of the main characteristics of the acute phase response involves the production of APP's. Extensive research in *in vitro* model sytems has revealed that hepatic APP gene expression is primarily regulated by cytokines. Although cytokine-mediated regulation of APP synthesis is highly complex, involving both stimulatory and inhibitory interactions between individual mediators, IL-6 appears to be a major regulator (2). Few data, though, are available on *in vivo* effects of cytokines on APP synthesis in humans. In chapter 3 we found that plasma IL-6 levels were correlated with CRP and transferrin levels, whereas the in chapter 6 observed time dependency between TNF and IL-6 profiles and the alterations in APP concentrations after ILP strongly suggests a causal relationship. According to *in vitro* studies (2), this modulation of APP production may primarily be mediated by IL-6, possibly potentiated by elevated cortisol levels, which were shown to parallel IL-6 levels in chapter 8. However, considering the redundancy of many cytokine actions, other mediators, e.g. of the IL-6 family, may influence APP synthesis as well.

Although the course and features of the acute phase response often follow a homologous pattern in different etiologies, it needs to be emphasized that some heterogeneity may exist in acute phase characteristics in various clinical conditions. For instance, increased synthesis of APP's is not always accompanied by leukocytosis and vice versa. In patients with both active and non-active SLE α1-acid glycoprotein levels are frequently elevated in contrast to CRP levels. In addition, the time courses of TNF and IL-6 levels in SLE appear to be dissociated, without a recognizable time dependency between these cytokines and changes in APP profiles

(12). Another form of an aberrant course of the acute phase response involves excessive serum amyloid A production and tissue deposition of amyloid in secondary amyloidosis accompanying infectious or inflammatory disorders (13). No data are available on the pathways regulating differential expression of acute phase phenomena. Furthermore, it is not known which mechanisms underlie transition of the acute phase response to the chronic inflammatory state. It might be speculated that fine tuning of the acute phase response and its eventual conversion to chronic inflammation are related to expression of different cytokine patterns. This hypothesis needs, however, further investigation. For instance by assessment of cytokine and APP profiles during chronic TNF or IL-6 administration in cancer patients. In such a model the sequence of pathogenetic factors in ACD could be studied as well.

9.3 Iron metabolism in the acute phase response and ACD

Disturbance of iron homeostasis, resulting in a shift of available iron to storage sites, plays a major role in the development of ACD. Iron redistribution to liver and MPS in inflammation is proposed to rely on an impaired tissue iron release due to entrapment of labile iron into an increased apoferritin pool (14). Proinflammatory cytokines are thought to be involved in the induction of this hypoferraemic response. This concept, however, is primarily based on studies in experimental animals and the effects of cytokines on iron metabolism in humans have not been investigated previously. In chapter 3 we found that TNF and IL-6 levels are correlated with parameters of iron status in RA patients with ACD and in chapter 7 it was observed that treatment of cancer patients with r-HuIFNγ and r-HuTNF is accompanied by changes in iron metabolism compatible with the alterations seen in inflammation and ACD. The induction of hypoferraemia was characterized by simultaneous inverse changes in serum ferritin, which appeared to act as an early APP, and serum iron levels. These findings support the concept that iron retention by liver and MPS is, at least in part, based on a primary stimulation of apoferritin synthesis by cytokines. In addition, an increased iron influx into storage compartments may be involved. As outlined in chapter 7, cytokine-controlled ferritin synthesis is complex and may be mediated at both transcriptional and translational level. The exact regulatory mechanism has, however, not been elucidated yet and future studies should focus on:

(a) Identification of involved transcription factors and their regulation.

(b) Modulation of the IRP-IRE-pathway by cytokines. For instance, *in vitro* IFNγ downregulates IRP activity in macrophages, associated with an increased ferritin synthesis (15,16). Similar experiments should be performed with other cell types, e.g. hepatocytes, and with other cytokines such as TNF. The *in vitro* findings may correlate with inflammation *in vivo* as in

monocytes obtained from patients with infectious and inflammatory disorders IRP activity is considerably decreased (16). It would be interesting to examine the sequential effects of TNF on monocyte IRP activity in a longitudinal study using the ILP model.

(c) Effects of cytokines on ferritin subunit distribution. Cytokines like TNF may preferentially stimulate synthesis of ferritin H-chains, which incorporate iron more efficiently than ferritin L-chains (17,18). However, whether a shift to H-chain-rich ferritin occurs in the acute phase response *in vivo* has not been established yet.

Thus, cytokines are likely to mediate the iron shift to storage sites in ACD. This mechanism may also underlie the development of functional iron deficiency in patients with chronic renal failure treated with haemodialysis. As a result, body iron stores are normal but iron supply to the bone marrow is inadequate, limiting the response to r-HuEPO treatment (19). This functional iron deficiency may in part be explained by proinflammatory effects of haemodialysis itself resulting in the release of cytokines like TNF and IL-1 (20,21). Indeed, an inflammatory state in dialysis-treated patients, reflected by high CRP levels, is associated with high ferritin levels, a decreased iron absorption and an impaired response to r-HuEPO (22). Thus in this subset of patients the syndrome of anaemia of chronic renal failure has also an ACD component.

Apart from iron redistribution to storage sites, an intrinsic defect in erythroid iron uptake capacity may contribute to the decreased iron availability for haeme synthesis in ACD. Previously, it was shown that transferrin binding and iron uptake by erythroblasts are reduced in RA patients with ACD (23). We speculated whether this could be related to alterations in erythroid TfR expression. In chapter 4 we found a reduced TfR number on erythroblasts in RA patients with ACD compared to nonanaemics and healthy subjects. This finding is surprising considering the presence of functional iron deficiency in ACD which is expected to lead to an upregulation of TfR synthesis via the IRP-IRE-pathway. Cytokines like TNF, IL-1 and IFNγ may downregulate erythroid TfR expression, possibly via modulation of IRP activity, as part of their suppressive effects on erythropoiesis in ACD. Indirect evidence for this concept is provided by the in chapter 7 observed decrease in sTfR concentrations after r-HuIFNγ and r-HuTNF administration in cancer patients. In addition, the impaired erythroid TfR expression may be related to the blunted EPO response in ACD. It is not known, however, whether a decreased erythroid TfR expression precedes the development of ACD, thereby contributing to the genesis of the anaemia, or rather is a consequence of an impaired erythropoiesis and thus an epiphenomenon. To differentiate between these possibilities erythroid TfR expression and iron uptake should be assessed before and during the induction phase of ACD. Iron uptake by erythroid precursors in ACD may further be diminished via inhibition of transferrin binding to its receptor by the APP α1-antitrypsin as has been shown *in vitro* (24). Whether α1-antitrypsin

is indeed a mediator of ACD *in vivo* remains speculative. In this respect, it would be interesting to examine transferrin-TfR interaction and erythroid iron uptake in patients with inherited α1-antitrypsin deficiency with and without ACD.

Thus, in ACD profound changes occur in iron handling by both storage and functional compartments. Iron transport between storage sites and the erythron is mediated by transferrin and we speculated whether changes in transferrin glycosylation, proposed to modulate its functional properties, are operative in ACD. In RA patients we found a shift in transferrin microheterogeneity towards highly sialylated fractions which was associated with disease activity-related parameters and the presence of ACD (chapter 3). We hypothesized on the possible functional consequences of alterations in transferrin glycosylation. First, an increase in highly sialylated transferrin variants in ACD may serve as a compensatory mechanism to facilitate iron transport to bone marrow as during pregnancy analogous changes occur in transferrin microheterogeneity which coincide with an increased iron transport to maternal bone marrow and placenta. However, this concept is not supported by current *in vitro* data, albeit to date only erythroid iron uptake from 4- and 6-sialo transferrins was studied. To obtain more insight in the role of transferrin glycosylation in ACD further study is needed on binding and internalization of these and other transferrin variants by various cell types, i.e. erythroblasts, hepatocytes and macrophages, obtained from healthy subjects but also from patients with ACD. Second, in the inflammatory state, an increased transferrin sialylation may counterbalance an enhanced clearance of partially desialylated transferrins by the upregulated hepatic asialoglycoprotein receptor system. For human TBG for instance it was shown that *in vivo* circulating half-lifes of fractions with a higher sialic acid content are significantly longer compared to their lower sialylated counterparts (25). Assessment of the *in vivo* half-lifes of purified transferrin isotypes in both physiological and inflammatory circumstances may indicate whether the clearance rate of transferrin is also dependent on its sialic acid content.

Having established a correlation between the change in transferrin microheterogeneity and TNF and IL-6 levels in the chronic inflammation model, i.e. RA, we examined the effects of acute inflammation, i.e. TNF administration, on transferrin glycosylation patterns in cancer patients (chapter 7). Total transferrin levels decreased after TNF infusion, presumably in part due to a reduced synthesis, no changes, though, were observed in transferrin subfraction distribution. This may be explained by the relatively short duration of increased systemic TNF and IL-6 levels. Further study on the possible effects of cytokines like TNF and IL-6 on transferrin glycosylation should include a model with chronic cytokine treatment and *in vitro* studies with primary human hepatocytes. Another approach would be to examine transferrin microheterogeneity patterns in RA patients treated with anti-TNF monoclonal antibodies.

9.4 Functional aspects of ACD

The alterations in iron metabolism and erythropoiesis in ACD are similar in a wide variety of clinical disorders, pointing to the existence of a final common pathway(s) in the pathogenesis of ACD. In this respect, our and other studies suggest that the induction of ACD and its associated changes in iron metabolism are mediated by the Th1 response via effects of Th1-type cytokines, i.e. TNF, IL-1 and IFNγ. Within this pathway, macrophages, as important effector cells, play a key role in the pathogenesis of ACD. First, by the release of proinflammatory cytokines, either systemically or locally in the bone marrow, which: (a) induce hypoferraemia; (b) inhibit transferrin synthesis; (c) suppress erythroid growth and TfR expression and (d) possibly inhibit the EPO response to the anaemia. Second, by modulation of iron processing resulting in a diversion of iron into increased apoferritin stores (Fig. 1).

What are the functional consequences of the changes in iron metabolism and the induction of ACD? Entrapment of iron into storage sites, based on redistribution of iron to liver and MPS and a decreased erythroid iron consumption, may serve a functional purpose (Table 2):

(a) The hypoferraemic response may be a non-specific host defense mechanism against microbial challenge and neoplastic growth as iron is an essential factor for the proliferation of pathogenic microorganisms and maligant cells (26). Although not always consistent, some evidence exists that moderate iron deficiency may protect against whereas iron overload may increase susceptibility for infectious disease (27,28).

(b) Diversion of iron into ferritin decreases the amount of "free" iron and may therefore be a protective mechanism against the "free" iron-catalyzed production of toxic hydroxyl radicals (29,30). However, disturbance of control mechanisms of iron homeostasis may be involved in various disease processes. For instance, in RA iron accumulates in the synovium which would, in turn, downregulate IRP activity and increase ferritin synthesis. However, in synovial polymorphonuclear cells IRP is found in its active form, possibly mediated by inflammatory substances like H_2O_2 and NO which may override the effect of iron on IRP activity (31). Treatment of RA patients with iron chelators may indeed reduce joint inflammation (32). Further study is needed on disregulation of iron homeostasis in various clinical disorders and the possible application of iron chelating agents.

(c) Iron may modulate immune effector functions. Low intracellular labile iron levels may favour cytokine responsiveness and cytokine production by monocytes/macrophages. For instance, *in vitro*, IFNγ-mediated stimulation of monocytes is inhibited in the presence of increased labile iron, but enhanced after iron chelation (33). Likewise, TNF production by iron-

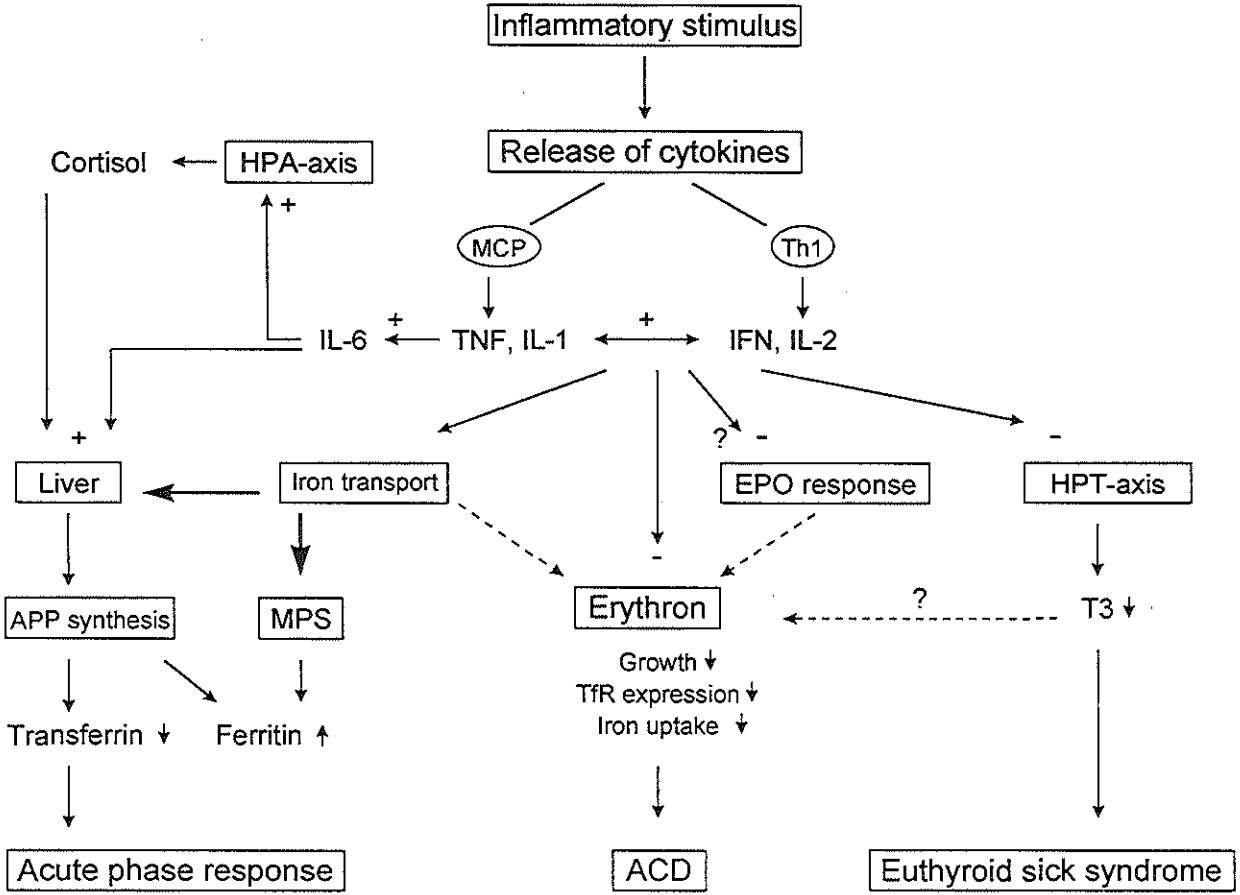
Inflammatory stimulus
Release of cytokines
Cortisol
HPA-axis
MCP
Th1
IL-6
TNF, IL-1
IFN, IL-2
Liver
Iron transport
EPO response
HPT-axis
APP synthesis
MPS
Erythron
T3
Growth
TfR expression
Iron uptake
Transferrin
Ferritin
Acute phase response
ACD
Euthyroid sick syndrome

Figure 1. Schematic representation of the pathogenesis of ACD. Abbreviations: ACD, anaemia of chronic disease; APP, acute phase protein; EPO, erythropoietin; HPA, hypothalamus-pituitary-adrenal; HPT, hypothalamus-pituitary-thyroid; IFN, interferon γ; IL, interleukin; MCP, macrophage; MPS, mononuclear phagocyte system; Th1, T helper 1 lymphocyte; TNF, tumor necrosis factor. ——— , increase; ------ , decrease.

loaded macrophages is decreased (34). Furthermore, phagocytic function of macrophages and their ability to kill intracellular pathogens is impaired in the presence of iron overload (35,36).

Table 2. Beneficial effects of hypoferraemia in acute and chronc inflammation.

* Iron deprivation from pathogenic microorganisms and tumor cells
* Prevention of excessive hydroxyl radical production
* Facilitation of macrophage cytokine responsiveness
* Facilitation of cytokine production by macrophages
* Favouring macrophage effector functions, e.g. phagocytosis

Thus, the hypoferraemic response has several beneficial effects in the host response to tissue injury of various origins, albeit at the cost of the development of anaemia. Another functional reason for the induction of ACD may be that inhibition of relatively nonessential anabolic processes, in casu erythropoiesis, may allow other metabolic reactions to occur in the chronic inflammatory state. In this respect we hypothesized on a possible relationship of ACD with the euthyroid sick syndrome (chapter 8). First, both ACD and the euthyroid sick syndrome frequently accompany a wide variety of chronic disorders. Second, ACD and the euthyroid sick syndrome share pathogenetic pathways as illustrated in chapter 8 showing the role of TNF and IL-6 in the induction of the euthyroid sick syndrome. The low T3 state is thought to counteract excessive catabolism although the exact metabolic consequences have not been established yet. As thyroid hormone exerts stimulatory effects on erythropoiesis, both directly and indirectly via an enhanced EPO production (37-39), it might be speculated that low thyroid hormone concentrations may contribute to the impaired erythropoiesis and blunted EPO response in ACD. Further study is needed to test this hypothesis. For instance, by assessment of the clinical association between ACD and the euthyroid sick syndrome in different etiologies, preferably in a longitudinal study. In addition, examination of bone marrow obtained from patients with the euthyroid sick syndrome but not (yet) ACD may indicate whether erythropoiesis is affected by the low T3 state.

9.5 Diagnostic aspects of ACD and the assessment of iron stores in inflammation

The differentiation between ACD and IDA is a common diagnostic problem in clinical practice. In particular the detection of co-existing iron deficiency [ID] in inflammatory states is often difficult as disproportionally increased serum ferritin levels can mask concomitant ID. Diagnostic accuracy of serum ferritin can be improved by increasing cut-off points for ID to values between 50 and 100 µg/l (40-42), although this is not a standardized method. Measurement of sTfR concentrations, which increase in ID, may be an additional tool in the assessment of iron stores in inflammatory states. Previously it was shown that the sTfR level can adequately differentiate uncomplicated IDA from ACD in which sTfR levels are normal to slightly increased (43,44). In chapter 5 we found that in patients with an inflammatory disorder, i.e. active RA, sTfR levels are significantly elevated in patients with depleted iron stores compared to patients with ACD, with both groups classified according to bone marrow iron content. This is in line with the study of Punnonen et al. who found similar results in patients with both inflammatory and infectious disorders (45). Thus, in the evaluation of anaemia in inflammatory conditions the combination of elevated sTfR and normal to increased ferritin levels points to a high probability of IDA, whereas the combination of normal sTfR and increased ferritin levels indicates the presence of ACD with a low probability of co-existing ID (Fig. 2). However, large studies in anaemic patients, classified according to bone marrow iron content, with disorders of various etiologies as well as standardization of the sTfR assay are needed to establish the role of the sTfR in the assessment of iron stores in inflammation.

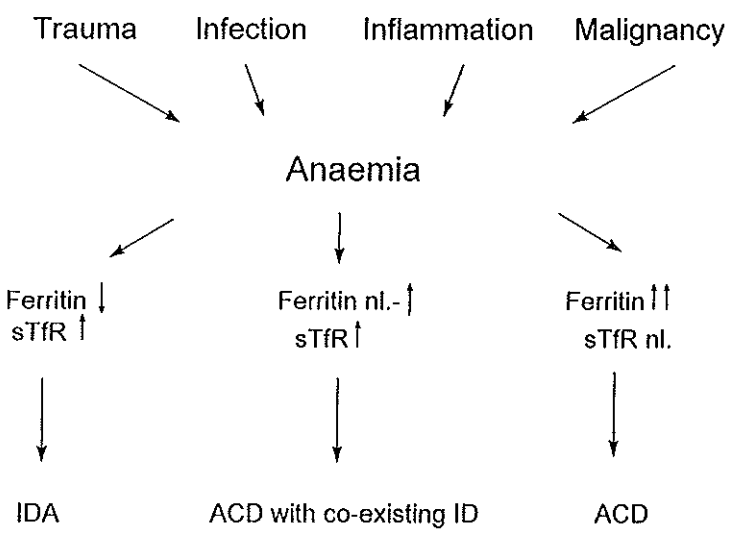

Figure 2. Classification of anaemia in the chronic inflammatory state according to serum ferritin and sTfR concentrations. IDA, iron deficiency anaemia; ACD, anaemia of chronic disease; sTfR, serum transferrin receptor; nl., values within the normal range.

Therapeutic aspects of ACD

ACD is a mild and nonprogressive anaemia and usually resolves during recovery of the underlying disease. Considering the possible beneficial effects of ACD and its associated changes in iron metabolism in chronic infection, inflammation and malignancy, the question arises whether there is a rationale for treatment of ACD. On the other hand, so far no harmful effects in this respect have been observed in studies in which ACD is corrected, e.g. by administration of r-HuEPO (46). Treatment of ACD may be indicated in the presence of serious co-existing cardiac or pulmonary pathology, perioperatively in case of major surgery and possibly in chronic disorders to improve quality of life (47,48). ACD can successfully be treated with r-HuEPO (46) and iron chelators (49). In chapter 5 we examined the effects of both treatment modalities on sTfR levels. Treatment of RA patients with ACD with r-HuEPO improved erythropoiesis with a parallel increase in sTfR levels. Measurement of sTfR levels may thus be a useful tool to monitor erythropoiesis. Indeed, the initial rise in sTfR levels in r-HuEPO treated patients with anaemia of chronic renal failure or anaemia associated with malignancy was shown to predict the ultimate haematological response (50,51). However, in patients on maintenance r-HuEPO therapy the sTfR may lose its specificity for detecting ID since an increased erythroid proliferation itself raises sTfR levels (52). We found that r-Hu-EPO treatment in RA results in iron mobilization reflected by a decrease in serum ferritin levels. The observed rise in sTfR levels may thus be explained by both an increased erythroid precursor cell mass and depletion of iron stores. Indeed, in the patients who became ID after r-HuEPO administration, sTfR levels were slightly higher. Therefore, detection of ID during r-HuEPO treatment requires simultaneous sTfR and ferritin measurements.

Treatment of RA patients with ACD with the iron chelator L1 was accompanied by a less pronounced but significant rise in sTfR levels paralleling a slight increase in Hb levels. This increase in sTfR levels may be explained by a reduction of iron stores and an increased erythroid TfR expression induced by the chelator itself and/or by an improved endogenous EPO response (49). Larger, long-term studies are needed to evaluate the effects of oral iron chelation treatment on erythropoiesis and iron metabolism in ACD in RA.

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Chapter 10

SUMMARY - SAMENVATTING

Summary

The acute phase response refers to a coordinated series of physiological changes in response to tissue injury. Stimuli which initiate the acute phase response can be of infectious, inflammatory, neoplastic or traumatic origin. The acute phase response is considered as an essential adaptive mechanism to maintain or restore homeostasis. Cytokines are thought to play a central role in the initiation and coordination of the acute phase response. In addition, endocrine changes, e.g. in the cortisol and thyroid hormone axis, may also participate in the regulation of the host response to tissue injury. Transition of the acute phase response to chronic inflammation is frequently accompanied by the development of anaemia, referred to as ACD. ACD is second to iron deficiency the most common cause of anaemia in hospitalized patients, its pathogenesis, however, is incompletely understood. Factors postulated to be involved include disturbance of iron metabolism, inhibition of erythropoiesis by cytokines, an impaired EPO responsiveness and a decreased erythrocyte survival.

In **chapter 1**, an overview is presented of the current concepts of the acute phase response and its regulation by the cytokine network, iron metabolism in health and inflammation and the pathophysiology of ACD. The proinflammatory cytokines TNF, IL-1, IL-6 and IFNγ play a major role in the modulation of APP synthesis and glycosylation, one of the major characteristics of the acute phase response. In addition, bidirectional interactions exist in the acute phase response between cytokines and endocrine systems, which are in particular discussed for cortisol and thyroid hormone metabolism. In inflammation, profound changes occur in iron fluxes between storage sites and erythron as well as in the synthesis of the iron binding proteins transferrin and ferritin. In addition, transferrin glycosylation is thought to be influenced by the acute phase response. These alterations in iron metabolism and the possible involvement of cytokines are discussed in detail. The development of ACD is related to the continuing inflammatory response and the release of proinflammatory cytokines. A review is given of the above mentioned pathogenetic mechanisms of ACD and the possible role of cytokines. Finally, diagnostic and therapeutic aspects of ACD are discussed.

The aim of this thesis was to obtain more insight in the regulation of the acute phase response and the pathogenesis of ACD with respect to the role of cytokines, endocrine systems and iron metabolism. Studies were performed in patients with RA as model for the chronic inflammatory state (Part I, chapter 3-5) and in cancer patients (melanoma, sarcoma) treated with ILP with r-HuTNF as model for the acute phase response (Part II, chapter 6-8).

In **chapter 2** patient characteristics and all used laboratory procedures are described.

In **chapter 3** iron status and transferrin microheterogeneity patterns were assessed in patients with RA in relation to disease activity, cytokine levels and the presence or absence of ACD. The changes in iron status in ACD, characterized by low serum iron and transferrin levels and elevated ferritin levels, correlated with serum TNF and IL-6 levels. In both anaemic (ACD and IDA) and nonanaemic RA patients a shift in transferrin microheterogeneity was observed towards fractions with an increased degree of glycan branching. This change in transferrin glycosylation was most pronounced in ACD and was correlated with disease activity-related parameters and increased levels of TNF and IL-6. Although highly speculative, modulation of transferrin glycosylation may serve as a compensatory mechanism to facilitate iron transport to erythroblasts in ACD or as a protective mechanism against an enhanced transferrin clearance.

In **chapter 4** the number and affinity of TfRs expressed by erythroblasts obtained from RA patients with and without ACD were examined. The number of TfRs on erythroblasts from patients with ACD was significantly lower as compared to nonanaemic patients and controls, whereas the affinity of the TfR tended to be lower in ACD. These preliminary data indicate that erythroid TfR expression might be impaired in ACD and may explain the previously observed decreased transferrin binding and iron uptake by erythroblasts in ACD in RA.

In **chapter 5** sTfR levels were measured in RA patients without anaemia and with IDA or ACD, classified according to bone marrow iron content. In addition, the effects were studied of treatment with r-HuEPO and the iron chelator L1 on sTfR levels in ACD. The sTfR level was significantly elevated in anaemic RA patients with depleted iron stores compared to nonanaemics and ACD. This might point to a role for the sTfR in the detection of iron deficiency in inflammatory states. Treatment with r-HuEPO improved erythropoiesis which was accompanied by an increase in sTfR and a decrease in ferritin levels. The rise in sTfR levels may be explained by both an increased erythroid proliferation and reduced iron stores. Treatment with L1 resulted in iron mobilization, indicated by decreased ferritin levels, and a slight increase in Hb values. This was also accompanied by an increase in sTfR levels which may reflect depletion of iron stores and/or an upregulated erythroid TfR expression.

In **chapter 6** ILP with r-HuTNF and melphalan in cancer patients was used as a model to examine various aspects of the acute phase response. We investigated the prolonged effects (7 days) of TNF perfusion on TNF, sTNF-R's, IL-6 and APP concentrations. After ILP, leakage

of TNF resulted in systemic peak levels followed by an increase in sTNF-R and IL-6 levels. Positive APP profiles showed a sequential time course with CRP representing the early APP response and α1-antitrypsin, α1-acid glycoprotein, C3 and C4 reflecting the second APP wave. The concentrations of albumin and transferrin, both negative APP's, decreased followed by a gradual recovery. It is concluded that TNF administration in humans initiates the acute phase response, presumably via the induction of IL-6. The simultaneous release of sTNF-R's may be part of the counter-regulatory mechanisms to control the inflammatory response.

In **chapter 7** we examined the role of TNF in the regulation of iron metabolism in the acute phase response by serial measurements of iron status, sTfR levels and transferrin microheterogeneity patterns in cancer patients treated with ILP with r-HuTNF. During pretreatment with r-HuIFNγ and after r-HuTNF administration serum iron and sTfR levels showed a marked decrease, associated with a simultaneous increase in serum ferritin levels, which parallelled CRP values. Transferrin subfraction distribution was not siginificantly altered by TNF administration. It is proposed that cytokines play a role in the modulation of iron metabolism in the acute phase response and ACD. TNF, possibly via IL-6, and IFNγ induce hypoferraemia, which may in part result from a decreased tissue iron release based on a primary stimulation of ferritin synthesis. The decrease in sTfR levels may reflect an impaired erythroid growth and/or TfR expression mediated by TNF and IFNγ. This may explain the relatively low sTfR levels as seen in ACD.

In **chapter 8** the effects of ILP with r-HuTNF were investigated on cortisol and thyroid hormone metabolism. ILP was followed by a rapid increase in cortisol levels, presumably mediated by TNF and IL-6. This cortisol response may potentiate the effects of cytokines on APP synthesis, but may also down-regulate endogenous TNF and IL-6 production. TNF administration induced, possibly via IL-6, profound changes in thyroid hormone concentrations, compatible with the euthyroid sick syndrome which is thought to counteract excessive catabolism in inflammation. Recovery of this euthyroid sick syndrome was characterized by a substantial and persisting increase in TSH levels which preceded the return of T3 and T4 to pretreatment levels. This may indicate that the recovery of the euthyroid sick syndrome is, at least in part, TSH-dependent.

In **chapter 9** the obtained results as described in chapter 3-8 are integrated and discussed with respect to their relevance for the understanding of the regulation of the acute phase response and the pathogenesis, diagnosis and treatment of ACD.

Chapter 10

Samenvatting

De acute fase reactie omvat een serie fysiologische veranderingen in respons op weefselbeschadiging. Stimuli die de acute fase reactie initiëren kunnen van zowel infectieuze, inflammatoire, maligne of traumatische origine zijn. De acute fase reactie wordt beschouwd als een essentieel aanpassingsmechanisme voor het handhaven of herstellen van de homeostase. Cytokinen spelen waarschijnlijk een centrale rol in de inductie en coördinatie van de acute fase reactie. Daarnaast zijn endocriene veranderingen, b.v. in de cortisol- en schildklierhormoon-as, mogelijk ook betrokken bij de regulatie van de acute fase reactie. De overgang van de acute fase reactie naar de chronische ontstekingsreactie gaat vaak gepaard met de ontwikkeling van een anaemie, gedefinieerd als ACD. Hoewel ACD, na ijzerdeficiëntie, de meest voorkomende anaemie is bij klinische patiënten is de ontstaanswijze van ACD nog onvoldoende opgehelderd. Factoren die mogelijk een rol spelen in de pathogenese van ACD zijn veranderingen in ijzermetabolisme, inhibitie van de erythropoëse door cytokinen, een verminderde EPO respons en een kortere erythrocytenoverleving.

In **hoofdstuk 1** wordt een overzicht gegeven van de huidige inzichten omtrent de acute fase reactie en diens regulatie door cytokinen, ijzermetabolisme in zowel fysiologische omstandigheden als tijdens de ontstekingsreactie en de pathophysiologie van ACD. De proinflammatoire cytokinen TNF, IL-1, IL-6 and IFNγ spelen waarschijnlijk een regulerende rol in de modulatie van acute fase eiwit synthese en glycosylering, één van de belangrijkste kenmerken van de acute fase reactie. Daarnaast bestaan er interacties tussen cytokinen en endocriene systemen in de regulatie van de acute fase reactie, welke met name worden besproken v.w.b cortisol en schildklierhormoon metabolisme. In acute en chronische ontstekingsreacties treden belangrijke veranderingen op in ijzertransport tussen opslagcompartiment en erythron alsmede in de synthese van de ijzerbindende eiwitten transferrine en ferritine. Daarnaast wordt de glycosylering van transferrine waarschijnlijk beïnvloed door de acute fase reactie. Deze veranderingen in ijzermetabolisme en de mogelijke rol van cytokinen hierin worden in detail besproken. De ontwikkeling van ACD is gerelateerd aan continuering van de acute fase reactie en het vrijkomen van proinflammatoire cytokinen. In dit perspectief wordt een overzicht gegeven van de bovengenoemde pathogenetische mechanismen van ACD. Tenslotte worden diagnostische en therapeutische aspecten van ACD besproken.

Het doel van onze studie was om de regulatie van de acute fase reactie en de pathogenese van ACD nader te bestuderen m.b.t. de rol van cytokinen, endocriene systemen en ijzermetabolis-

me. Hiervoor werden studies verricht bij patiënten met RA als model voor de chronische ontstekingsreactie en ACD (deel I, hoofdstuk 3-5) en bij kankerpatiënten (melanoom, sarcoom) die werden behandeld met geïsoleerde regionale perfusie ['isolated limb perfusion', ILP] met r-HuTNF als model voor de acute fase reactie (deel II, hoofdstuk 6-8).

In **hoofdstuk 2** worden patiënten kenmerken beschreven alsmede alle laboratoriumtechnieken zoals toegepast in de studies van dit proefschrift.

In **hoofdstuk 3** werden de ijzerstatus en transferrine microheterogeniteitspatronen onderzocht in patiënten met RA in relatie tot ziekteactiviteit, cytokine concentraties en de aan- of afwezigheid van ACD. De veranderingen in ijzerstatus in ACD, gekarakteriseerd door lage serum ijzer en transferrine waarden en verhoogde ferritine waarden, bleken gecorreleerd te zijn met serum TNF en IL-6 concentraties. Bij zowel anaemische (ACD en IDA) als niet-anaemische RA patiënten werd een verschuiving in het transferrine microheterogeniteitspatroon waargenomen resulterend in een toename van de fracties met sterk vertakte koolhydraatketens. Deze verandering in transferrine glycosylering was het meest uitgesproken in ACD en was gecorreleerd met ziekteactiviteit-gerelateerde parameters en toegenomen TNF en IL-6 concentraties. Geconcludeerd wordt, zij het speculatief, dat de modulatie van transferrine glycosylering in ACD mogelijk dient als compensatoir mechanisme ter bevordering van ijzertransport naar erythroblasten en/of ter preventie van een verhoogde transferrine klaring in de acute fase reactie.

In **hoofdstuk 4** werd onderzocht of ACD in RA gepaard gaat met veranderingen in erythroïde TfR expressie m.b.t. TfR aantal en affiniteit. Bij RA patiënten met ACD werd een significant lager aantal TfR per erythroblast gevonden vergeleken met niet-anaemische RA patiënten en met gezonde individuen. Daarnaast bleek de affiniteit van erythroïde TfR lager in RA patiënten met ACD, hoewel niet significant, vergeleken met niet-anaemische RA patiënten en met de controlegroep. Deze preliminaire gegevens wijzen op een verminderde erythroïde TfR expressie in ACD hetgeen de reeds eerder vastgestelde verminderde transferrine binding en ijzeropname door erythroïde voorlopercellen in ACD kan verklaren.

In **hoofdstuk 5** werden sTfR concentraties bepaald bij RA patiënten zonder anaemie en bij RA patiënten met ijzergebreksanaemie en ACD, geclassificeerd o.b.v. kleurbaar ijzer in het beenmerg. Daarnaast werd onderzocht wat de effecten zijn van behandeling van ACD bij RA met respectievelijk r-HuEPO en de ijzerchelator L1 op sTfR waarden in relatie tot veranderingen in

erythropoëse en ijzerstatus. Bij RA patiënten met ijzerdeficiëntie werd een significant hogere sTfR concentratie gevonden vergeleken met RA patiënten zonder anaemie of met ACD. Dit suggereert dat bepaling van de sTfR concentratie mogelijk een rol kan spelen bij de detectie van ijzerdeficiëntie bij inflammatoire aandoeningen en de differentiatie met ACD. Behandeling met r-HuEPO resulteerde in een verbetering van de erythropoëse welke gepaard ging met een toename in de sTfR en een afname in de ferritine concentratie. De sTfR stijging kan verklaard worden door zowel een toename in erythroïde proliferatie als door een reductie van de lichaamsijzervoorraad. Behandeling met L1 leidde tot ijzermobilisatie, weergegeven door een daling in ferritine, alsmede tot een geringe Hb stijging. Ook hierbij werd een toename in sTfR concentraties waargenomen die mogelijk depletie van ijzervoorraden en/of een toegenomen erythoïde TfR expressie reflecteert.

In **hoofdstuk 6** werd de behandeling van kankerpatiënten met ILP met r-HuTNF en melphalan gebruikt als model om diverse aspecten van de acute fase reactie te bestuderen. Onderzocht werd het longitudinale effect van TNF-perfusie op serum TNF, sTNF-R, IL-6 en acute fase eiwit concentraties. Resorptie van TNF na ILP resulteerde in een systemische TNF piek gevolgd door een toename in sTNF-R en IL-6 concentraties. Het profiel van de positieve acute fase eiwitten toonde een sequentieel beloop met een snelle stijging van CRP, als representant van de vroege acute fase eiwit respons, gevolgd door een toename van de zgn. late acute fase eiwitten α1-antitrypsine, α1-zure glycoproteïne en de complement factoren C3 en C4. De concentraties van albumine en transferrine, beide negatieve acute fase eiwitten, daalden na TNF-perfusie, gevolgd door een geleidelijk herstel. Geconcludeerd wordt dat TNF-toediening in een humaan model de acute fase reactie initieert, waarschijnlijk via de inductie van IL-6. De gelijktijdige toename in sTNF-R concentraties is mogelijk onderdeel van antiinflammatoire mechanismen die de progressie van de acute fase reactie antagoneren.

In **hoofdstuk 7** werd de rol onderzocht van TNF in de regulatie van ijzermetabolisme in de acute fase reactie door seriële bepalingen van ijzerstatus, sTfR concentraties en transferrine microheterogeniteitspatronen in kankerpatiënten behandeld met ILP met r-HuTNF. Gedurende de voorbehandeling met r-HuIFNγ én na TNF toediening trad een substantiële daling op van serum ijzer- en sTfR concentraties. Tegelijkertijd trad een stijging op van het serum ferritine met een parallel tijdsbeloop als CRP. Er werd geen effect gevonden van TNF-perfusie op de transferrine subfractie verdeling. Geconcludeerd wordt dat cytokinen waarschijnlijk een rol spelen in de modulatie van het ijzermetabolisme in de acute fase reactie en ACD. TNF, mogelijk via IL-6, en IFNγ induceren hypoferraemie die waarschijnlijk het gevolg is van een vermin-

derde ijzerafgifte door het opslagcompartiment o.b.v. een primaire stimulatie van ferritine synthese. De daling in sTfR concentraties reflecteert mogelijk een afname in erythroïde proliferatie en/of -TfR expressie, gemedieerd door TNF en IFNγ. Dit zou een verklaring kunnen zijn voor de relatief lage sTfR concentraties zoals gemeten in ACD.

In **hoofdstuk 8** werden de effecten onderzocht van ILP met r-HuTNF op cortisol en schildklierhormoon metabolisme. TNF-perfusie werd gevolgd door een snelle toename in cortisol waarden, waarschijnlijk geïnduceerd door TNF en IL-6. Deze cortisolrespons potentieert mogelijk de effecten van cytokinen op de acute fase eiwit synthese, maar heeft waarschijnlijk ook een inhiberend effect op de endogene TNF en IL-6 productie. TNF-toediening induceerde, mogelijk gedeeltelijk via IL-6, duidelijke veranderingen in schildklierhormoonconcentraties compatibel met het euthyroid sick syndrome. Dit euthyroid sick syndrome speelt mogelijk een rol bij de preventie van excessief catabolisme tijdens de acute fase reactie. Herstel van het euthyroid sick syndrome werd gekarakteriseerd door een substantiële en persisterende toename in de TSH concentratie die voorafging aan de normalisatie van T3 en T4 waarden. Dit suggereert dat het herstel van het euthyroid sick syndrome, in ieder geval gedeeltelijk, TSH-afhankelijk is.

In **hoofdstuk 9** worden de verkregen resultaten zoals beschreven in hoofdstuk 3-8 geïntegreerd en wordt de relevantie besproken van deze resultaten m.b.t. het inzicht in de regulatie van de acute fase reactie en de pathogenese, diagnostiek en behandeling van ACD.

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