ANEMIA IN RHEUMATOID ARTHRITIS

The role of iron, erythropoietin and cytokines

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ANEMIE BIJ RHEUMATOÏDE ARTHRITIS

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"Jongens, het leven is een vreemde reis,
maar wellicht leert een mensch wat onderweg".

(Uit: "De Jongen", Martinus Nijhoff, 1917)

Aan mijn ouders
Voor Annemarie en ...!

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The results of this thesis are set down in the following articles and abstracts

ARTICLES

Review. Anaemia in rheumatoid arthritis: pathogenesis, diagnosis and treatment.
G. Vreugdenhil and A.J.G. Swaak.
Rheumatology International 9:243-257, 1990

Anaemia in rheumatoid arthritis. The role of iron, vitamin B12 and folic acid deficiency and erythropoietin.
G. Vreugdenhil, A.W. Wognum, H.G. van Eijk and A.J.G. Swaak.
Annals of Rheumatic Diseases 1990, 29:(in press).

Anaemia of chronic disease: Diagnostic significance of erythrocyte and serological parameters in iron deficient rheumatoid arthritis patients.
G. Vreugdenhil, J.A.M. Baltus, H.G. van Eijk and A.J.G. Swaak.
British Journal of Rheumatology 1990, (in press).

Prediction and evaluation of the effect of iron treatment in anaemic RA patients.
G. Vreugdenhil, J.A.M. Baltus, H.G. van Eijk and A.J.G. Swaak.
Clinical Rheumatology 8(3):352-362, 1989.

Impaired iron uptake and transferrin binding by erythroblasts in anaemia in rheumatoid arthritis.
G. Vreugdenhil, M.J. Kroos, H.G. van Eijk and A.J.G. Swaak.
British Journal of Rheumatology, 1990, (in press).

Impaired erythropoietin responsiveness to the anaemia in rheumatoid arthritis. A possible inverse relationship with iron stores and effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one.
G. Vreugdenhil, G.J. Kontoghiorghes, H.G. van Eijk and A.J.G. Swaak.
Submitted.

Efficacy and safety of the oral iron chelator L1 in anaemic RA patients.
G. Vreugdenhil, G.J. Kontoghiorghes, H.G. van Eijk and A.J.G. Swaak.
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Tumor necrosis factor alpha as a cause of anemia in patients with rheumatoid arthritis: elevated serum levels and in vitro suppression of erythropoiesis.
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ABSTRACTS/PRESENTATIONS

First International symposium on oral iron chelation in the treatment of thalassaemia and other diseases. (Royal Free Hospital, London, United Kingdom, November 2-4, 1989).

Efficacy and safety of the oral iron chelator L1 in anaemic RA patients.
G. Vreugdenhil, G.J. Kontoghiorghes, H.G. van Eijk and A.J.G. Swaak.

Increase in haemoglobin and serum erythropoietin after oral iron chelation in anaemic RA patients.
G. Vreugdenhil, G.J. Kontoghiorghes, H.G. van Eijk and A.J.G. Swaak.

Internistendagen (Veldhoven, March 29-30, 1990)

Tumor necrosis factor alpha and anaemia in rheumatoid arthritis.
G. Vreugdenhil, B. Löwenberg, H.G. van Eijk and A.J.G. Swaak.

Defective erythroblast iron uptake in rheumatoid arthritis and anaemia.
G. Vreugdenhil, M.J. Kroos, H.G. van Eijk, B. Löwenberg and A.J.G. Swaak.

LIST OF ABBREVIATIONS

ACD = anemia of chronic disease
amol = attomol

B12 = vitamin B12
BFUe = burst forming units of erythroblasts

CRP = C-reactive protein
Clqba = Clq binding assay
c.o.p. = cut off point

dl = deciliter

ESR = erythrocyte sedimentation rate
Epo = erythropoietin
E-ferritin = erythrocyte ferritin
EIT = effective iron turnover

Fe = iron
fl = femtoliter

g = gram

Hb = hemoglobin
Ht = hematocrite
HBSS = Hanks balanced salt solution

IFN = interferon
IIT = ineffective iron turnover
IL-1 = interleukin-1
IL-2 = interleukin-2
IL-3 = interleukin-3
IL-6 = Interleukin-6

LEM = leucocyte endogenous mediator
L1 = 1,2-dimethyl-3-hydroxypyrid-4-one

MCH = mean corpuscular hemoglobin
MCV = mean corpuscular volume
MM/h = millimeter per hour
MIT = marrow iron turnover

nmol = nanomol
NSAID = nonsteroidal anti-inflammatory drugs

pg = picogram
$PGE_{1,2}$ = prostaglandin $E_{1,2}$
pmol = picomol

RA = rheumatoid arthritis

s- = serum-

TIBC = total iron binding capacity
TNFα = tumor necrosis factor alpha

μg = microgram
μmol = micromol

CHAPTER 1

ANEMIA IN RHEUMATOID ARTHRITIS: A REVIEW OF PATHOGENESIS, DIAGNOSIS AND TREATMENT

1.1 INTRODUCTION

Anemia frequently is a concomitant feature in chronic disease. It is often related to the underlying disease like in chronic renal failure, in which anemia develops by means of a decreased erythropoietin production. Other examples are gastrointestinal bleeding caused by a malignant, infectious or inflammatory process, hemolysis in inflammatory disorders and malignancy, malignant bone marrow infiltration and the various causes of (chronic) disease associated with hematuria, menorrhagia and hemolysis. Anemia can also be an indirect complication of the underlying disease, e.g. by means of poor nutrition or toxic side effects of drug therapy.

Apart from these causes a specific type of anemia is associated with chronic infection, inflammation, malignancy and autoimmune disorders (Table 1) termed the anemia of chronic disease (ACD). Cartwright did extensive research on the subject and defined the following criteria necessary for the diagnosis of ACD [1,2,3]: 1. decreased serum iron, 2. normal or decreased total iron binding capacity (TIBC), 3. decrease in iron saturation of transferrin, 4. decreased number of sideroblasts and increased stainable iron content in bone marrow.

The anemia develops slowly and nonprogressively within the first month of the underlying disease and its severity is to some extent correlated with disease activity [3]. After one month's duration of the underlying disorder the hemoglobin (Hb) level is constant and independent of disease duration. The Hb rarely reaches a level below 70% of normal in uncomplicated cases. Generally the cellular indices remain normal, but mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) can be low, even without iron deficiency [4]. The reticulocyte count is low for the degree of anemia.

ACD also occurs frequently in patients with rheumatoid arthritis (RA) [5,6]. In general it is not different from ACD associated with other disorders (Table 1) and it develops in most patients with RA of a certain disease activity. In active RA, however, concomitant iron

*TABLE 1 Diseases associated with ACD**

AUTOIMMUNE DISORDERS	CHRONIC INFECTIONS
RHEUMATOID ARTHRITIS SYSTEMIC LUPUS ERYTHEMATOSUS POLYMYOSITIS & DERMATOMYOSITIS SCLERODERMA VASCULITIS	FUNGAL BACTERIAL endocarditis osteomyelitis pyelonephritis decubitus abcesses tuberculosis
NEOPLASIA	**CHRONIC INFLAMMATORY DISORDERS**
SOLID TUMORS M. HODGKIN NON-HODGKIN LYMPHOMAS	SARCOIDOSIS M. CROHN ULCERATIVE COLITIS

* Fitting Cartwright's criteria; ref. 1-3. Other causes of anemia mentioned in the text are excluded

deficiency is reported in a frequency of 30-70% of anemic subjects [7-12].

The purpose of this study is to summarize all data available from the literature on pathogenesis, diagnosis and treatment of ACD and to formulate study aims for this thesis as an effort to solve the many controversies which exist in this field. The following aspects of ACD will be dealt with:

1.2 <u>Pathogenesis</u>

1.3 <u>Factors associated with other types of anemia than ACD in RA</u>

1.4 <u>Factors associated with ACD in RA</u>

- 1.4.1 iron absorption
- 1.4.2 mononuclear phagocyte system
- 1.4.3 ferritin and lactoferrin
- 1.4.4 ineffective erythropoiesis
- 1.4.5 mediators of erythropoiesis
- 1.4.6 hemolysis and red cell life span
- 1.4.7 erythropoietin
- 1.4.8 erythroid progenitor cells

1.5 <u>Functional aspects of ACD</u>

1.6 <u>Diagnosis</u>

1.7 <u>Treatment</u>

1.2 PATHOGENESIS

In RA many causes of anemia can be present apart from ACD. It can be the result from side effects of drugs or other secondary phenomena associated with RA. Some specific causes of other types of anemia tend to occur more frequently in RA than in a control population. The pathogenesis of these types of anemia is clearly different from ACD. Factors associated with ACD are a disturbed iron metabolism, an ineffective or inhibition of erythropoiesis, hemolysis, leucocyte endogenous mediators and decreased levels or decreased responsiveness to erythropoietin.

Most studies on the pathogenesis of ACD have been carried out in RA patients, so that some of the pathogenetic factors may be more specific for ACD in RA than for ACD as a complication of other chronic diseases.

1.3 FACTORS ASSOCIATED WITH OTHER TYPES OF ANEMIA THAN ACD IN RA

Gastrointestinal blood loss, occult or overt, may be seen in a higher frequency due to antirheumatic drugs like NSAIDs [13] and corticosteroids [14,15]. The increased ulcer prevalence in RA may be caused by an increased permeability of gastric mucosa for hydrogen ions [16].

Anemia in RA can also be the result of myelotoxic effects of gold, including aplastic anemia, [17,18] penicillamin [19] and cytostatic drugs [20].

Autoimmune phenomena can occur in RA leading to destruction of erythrocytes or thrombocytes (with consequent bleeding tendency sometimes) [21]. Cases of pure red cell aplasia have been described in the course of RA [22].

Another contributing factor may be an increased plasma volume in RA, especially among women [23], and salt retention due to aspirin [23] which causes hemodilution with a slight anemia as a consequence.

A number of vitamin deficiencies are found in patients with RA. Controversy exists about the occurrence of vitamin B12 deficiency. Bieder [24] found an increased frequency of pernicious anemia in RA,

but this was not confirmed by Ghazi [25]. Defective B12 resorption is only of clinical importance in a minority of cases, although gastric antibodies and gastric mucosal atrophy occur in a higher than normal frequency in RA [26].

Folic acid deficiency is also reported to be more frequent in RA [27], although megaloblastic changes are rare and possibly masked by other deficiencies [28]. Anorexia and increased utilization by proliferating synovial cells may be causes of folic acid deficiency [27,29].

Anemic RA patients are often vitamin C deficient, especially when using aspirin [30]. It is suggested that this deficiency might inhibit iron release from the mononuclear phagocyte system [31], resulting in hemosiderosis [32]. No clinical features of vitamin C deficiency are seen in these RA patients, however.

Copper-deficiency, known to inhibit erythropoiesis [33], is not encountered in RA patients, in fact it is often elevated [34]. Sometimes a decreased serum pyridoxin is found in RA without signs of sideroblastic anemia, however [35,36,37].

It is clear that in RA frequently more than one cause of anemia may be present simultaneously. This implies that studies examining one specific pathogenetic factor in anemia in RA should be interpreted with care.

1.4 FACTORS ASSOCIATED WITH ACD IN RA

1.4.1 Iron absorption (Table 2)

It was found by Roberts et al. [38] that iron absorption in anemic RA patients was disturbed unless concomitant iron deficiency was present. Later findings showed that iron absorption in RA patients is not different from controls [39]. Weber et al. did extensive research in the field of absorption, retention and utilization of iron in RA patients [40]. RA patients and iron deficient controls were given oral radiolabeled ^{59}Fe after which activity was measured in blood samples. It was found that mucosal uptake of iron in RA patients was the same as in controls but iron deficient RA patients had a lower uptake than

iron deficient patients without chronic disease. Mucosal transfer was lower in RA patients compared to controls and was higher when patients were iron deficient. No direct correlation was seen between disease activity and iron absorption. In another recent study [41], using the same method, it was shown that patients with active RA and anemia have an impaired iron absorption, whereas RA patients without anemia had normal iron absorption. Boddy [39] and Weber [40] found a lower rise of iron absorption in iron deficient RA patients compared to iron deficient patients without chronic disease, but Benn [41] showed that the rise was maximal.

The cause of increased retention and diminished absorption of iron in active RA remains unclear. A decrease in circulating transferrin with less free binding sites and iron trapping by submucosal intestinal tissue macrophages [42] might reduce iron transport to bone marrow. These findings are present in RA and ACD but not in iron deficient patients without chronic disease and may thus play a role in pathogenesis of ACD.

In our opinion two major factors underly the conflicting data presented in these studies. Although Cartwrights definition of ACD is quite clear it must be noted that iron deficient RA patients also have a certain level of disease activity. If iron is administered to them the mononuclear phagocyte system (MPS) will also tend to retain iron (see Table IIb) as it will in ACD. Therefore RA patients with iron deficiency can not fully be separated from those with ACD resulting in either masking of differences or conflicting data concerning iron absorption. Another problem is that iron samples taken from blood are the net result of iron absorption and utilization but also from retention and release by the MPS. If the gastrointestinal MPS retains iron it could be interpreted as decreased iron absorption, whereas iron is also extracted from blood and retained by the MPS. Therefore in active disease measuring iron absorption remains a difficult problem. Since in ACD -by definition- iron stores are increased we believe that a decreased iron absorption is rather the result of active RA than a cause of ACD or iron deficiency.

TABLE 2 Iron metabolism in ACD

	POSITIVE OBSERVATION	ROLE IN ACD	NEGATIVE OBSERVATION
DEFECTIVE IRON ABSORPTION	38,40,41*	- - + - -	39,41*
DEFECTIVE IRON RELEASE BY			
a) mononuclear phagocyte system	47,48,49,52	+ + / ? ?	11,50,51
b) ferritin	61	?	51
c) lactoferrin	66	?	
INEFFECTIVE IRON TURNOVER	67,68,69,72	+ + + +	70,71

A positive observation means that the factor involved was present whereas a negative observation means that the factor was absent. (- = positive observation/no role in ACD; + = positive observation/ role in ACD; ? = doubtful). Numbers reflect references cited.

* In this study a normal rise was found in iron deficiency whereas in ACD iron absorption was decreased

1.4.2 The role of the mononuclear phagocyte system (MPS) (Table 2)

Iron must be incorporated into erythroblasts for haemsynthesis and hence hemoglobin production. Generally, two ways exist for transport to the developing erythroblast. If intravenous iron dextran is administered it will be directly cleared by the MPS (formerly called reticuloendothelial system; RES) after which retention or liberation into the circulation takes place as transferrin bound iron being utilized by erythroblasts [43]. It is postulated that a certain amount of iron is transported from the MPS to bone marrow as ferritin (rhopheocytosis) but this plays a minor role [44]. The second and most important iron source for erythroblasts is transferrin bound iron released by the MPS after the breakdown of hemoglobin from the erythrocytes [45]. Agreement exists considering the fact that the first route, i.e., release of iron by MPS with iron dextran as a source is not disturbed in RA. Ferrokinetic studies with ^{59}Fe labeled transferrin showed normal erythrocyte incorporation in patients with RA and anemia after intravenous radiolabeled iron [46], while the same

author saw a diminished reutilization of iron after administering ^{59}Fe hemoglobin [47] which was confirmed by Beamish [48] who used a double isotope method; ^{55}Fe labeled dextran Hb was applied for measuring iron reutilization and ^{59}Fe transferrin for plasma iron utilization. Bennet [49] found that ^{59}Fe labeled dextran in RA patients was readily taken up by liver MPS but little mobilization occurred from it, except in a patient with concomitant iron deficiency. Williams [50] found, in contrast, no differences in MPS iron release between RA patients with and without ACD as well as normal controls, using a method which measures the conversion from ^{59}Fe dextran to ^{59}Fe transferrin although an unphysiological high dose was given. Bentley et al. [51] using ^{59}Fe hydroxide colloid as substrate, confirmed this in 7 out of 9 patients.

Studies on MPS iron release show the same controversy as was met in those considering iron absorption. It is obvious that the problem of overlap between iron deficiency and ACD is a major problem. In our opinion these studies should be repeated. Iron deficient RA patients should be separated more clearly from those with ACD, not only using stainable bone marrow iron as a parameter, which is a semiquantitative scale, but also using serum ferritin which reflects iron stores [8]. Furthermore, the iron deficient RA patients should be suppleted after which iron release must be measured again. The same holds true for those with ACD before and after antirheumatic treatment. This approach will eventually establish defective MPS iron release as a possible specific pathogenetic factor in ACD.

1.4.3 The role of ferritin and lactoferrin (Table 2)

It was formerly thought that apoferritin synthesis was regulated by the amount of nonstorage cellular iron [52], but Konijn and Hershko [53] found that ferritin synthesis preceded a fall in plasma iron after an immunologic stimulus like the injection of turpentine resulting in abcess formation. A larger proportion of iron entering the cell will be bound by apoferritin, the synthesis of which is augmented in inflammation [53], explaining the hypoferremia. Serum ferritin reflects tissue ferritin [54]. In acute infections the rise starts within 24-30 hours [55]. It probably reflects the acute phase reaction in inflammation [56].

Both ferritin [57] and iron content [58] are elevated in synovial fluid from RA patients. Ferritin is probably able to stimulate local free radical damage in joints by forming hydroxyl radicals [59], caused by iron release from ferritin, initiated by O_2^-. The free radical damage might contribute to the persistence of the synovitis [60]. Macrophages in RA have increased affinity for iron and they are a major component of the synovial membrane while RA synovium might have an increased iron avidity [61]. It might therefore be possible that these factors and the increased storage iron bound to apoferritin result in a decreased iron availability for and incorporation into erythroblasts, suggesting a role in the pathogenesis of ACD.

Lactoferrin is (like transferrin) an iron binding protein. It is mostly located within granules in neutrophilic leucocytes [62] and concentrations of it are high in inflammatory sites [63]. Lactoferrin release is mediated by leucocyte endogenous mediators [64]. Its affinity for iron is higher than transferrin [65]. Lactoferrin and iron are taken up easily by macrophages [66]. The result can be hypoferremia and decreased iron availability for transport to erythroblasts. Hansen [11] however, found that serum level of lactoferrin in anemic RA patients is not different from controls. This does not exclude a role of extravascular lactoferrin in the genesis of hypoferremia and ACD in these patients.

Iron trapping by the iron binding proteins ferritin and lactoferrin resulting in a decreased iron availability for bone marrow might be a contributive factor in the pathogenesis of ACD.

As stated in paragraph 1.4.2. these measurements should be done both transversally (comparing nonanemics, iron deficient patients and patients with ACD) and longitudinally (before and after antirheumatic treatment).

1.4.4 Ineffective erythropoiesis (Table 2)

It was found by Samson et al. [67] that in a patient with RA and ACD ineffective erythropoiesis was increased and returned to normal after antirheumatic therapy. They used a method which assesses ineffective erythropoiesis by measuring production of labeled bilirubin coming from the breakdown of red cell precursors. This

finding was confirmed by others who found ineffective iron turnover (IIT) in 6 of 11 patients with RA and ACD, using a ferrokinetic study based on the disappearance curve of ^{59}Fe labeled transferrin which reflects iron reflux from bone marrow into plasma [68]. In this manner iron incorporation into circulating red cells is called effective iron turnover (EIT). The sum of the two is total marrow iron turnover (MIT). This method was originally described by Rickets et al. [69]. It was shown in later studies, however, that IIT increase is in fact more specific for concomitant iron deficiency in ACD [70,71].

Another approach was to culture erythroblasts in vitro and measure the release of ^{59}Fe haem from them. It was found that the release of ^{59}Fe haem was significantly higher in RA patients with ACD compared to nonanemic RA patients, while it decreased after antirheumatic drug treatment and a correlation existed between ^{59}Fe haem release and disease activity [72].

IIT was supposed to be increased in ACD [67]. In other studies IIT turned out to be related to iron deficiency. This clearly demonstrates once again that in these studies patients' iron status should be properly defined. The in vitro studies measuring haem release from cultured erythroblasts point to a role of IIT in ACD. It could, however, also be argued that a higher percentage of erythroblasts die in vitro, resulting in increased haem release. We therefore think that a role of IIT in ACD can only be proven if patients are well defined and the methods sufficiently standardized. The 2 different approaches should be evaluated in the same patients before and after antirheumatic or -in case of iron deficiency- iron treatment.

1.4.5 Mediators of erythropoiesis (Table 3)

Many mediators have been described with both stimulatory and inhibitory effects on bone marrow cells.

Acidic isoferritins were able to suppress in vitro growth of granulocyte and macrophage progenitor cells [73,74,75]. They were shown to be released from leukemic cells suggesting a role of bone marrow depression in leukemia. Their role in erythropoiesis and ACD is not yet fully established.

Prostaglandins are important mediators of erythropoiesis.

Prostaglandin E_1 (PGE_1) stimulated erythroid colony growth but inhibited granulocyte and macrophage progenitor cells [76]. PGE_2 however, did not affect erythropoiesis in vitro [77]. Arachidonic acid metabolites in the lipoxygenase pathway are essential for erythroid colony growth which was confirmed by the observation that inhibitors of this pathway reduced erythropoiesis. Inhibitors of the cyclo-oxygenase pathway in contrast (such as aspirin) did not affect erythropoiesis [78]. Indomethacine, a PG synthetase inhibitor, did not influence erythropoietin induced erythroid colony growth [77]. Considering these findings it is very hard to investigate the effects of NSAID's, commonly used in RA treatment, on erythropoiesis in vivo and in vitro in RA patients with ACD, because the effects are different and not fully restricted to one certain pathway.

Serum interferon (IFN) gamma levels were elevated in aplastic anemia, whereas anti-IFN gamma addition in bone marrow cultures from these patients restored bone marrow cell growth [79]. IFN gamma suppressed erythroid colony growth [80] in pure red cell aplasia, whereas IFN alpha stimulated erythroid colony growth in myelodysplasia [81]. Peripheral blood lymphocytes were shown to produce decreased amounts of IFN gamma in RA patients [82]. This suggests that IFN gamma might be of no pathogenetic importance in ACD in RA patients, although in this study [82] the authors did not examine parameters of erythropoiesis.

It was found that a substance in sterile peritoneal exsudates in rabbits was able to cause fever. This was first called leucocyte pyrogen [83]. Later it was discovered that this substance was able to induce neutrophil release from bone marrow and synthesis of acute phase reactants like fibrinogen, ceruloplasmin, c-reactive protein, haptoglobin and others [83,84]. Because of its various biologic activities it was then called leucocyte endogenous mediator (LEM). It was also found that it was presumably produced in macrophages and that it exerted activating effects on lymphocytes after which it was called interleukin-1 (IL-1) [85].

It is known to produce degranulation in leucocytes with release of lactoferrin as a consequence [64]. Serum iron remained low during daily injections of LEM; possibly it was trapped by lactoferrin [86], resulting in a lower iron availability for erythroblasts.

Theoretically it is possible that LEM inhibits erythropoietin production because it is known to inhibit the biosynthesis of various proteins [85,87]. It was shown that IL-1 production by monocytes was higher in case of exacerbation of RA activity [88]. Schooley et al. [77] found that IL-1 was able to suppress erythropoietin-stimulated erythroid colony growth in vitro in mice. This effect was seen in both anemic and nonanemic mice, although less pronounced in the latter. Maury et al. [89] also showed that IL-1 inhibited erythroid colony growth while he saw that serum IL-1 was lower in nonanemic than in anemic RA patients. It was concluded that IL-1 could play a role in the pathogenesis of anemia in RA. Others however [90], found a lower monocyte IL-1 production in peripheral blood from RA patients compared to controls, making its role in vivo in RA and ACD less certain.

IL-2 production by T lymphocytes was shown to be inhibited by gold salts in vitro, the gold-concentration used being similar to the in vivo situation [91]. IL-2 was shown to reduce erythoid colony count [92]. In this way a possible direct or indirect role in the pathogenesis of ACD could be argued. However, IL-2 production by synovial cells with and without stimulation by phytohemagglutinin was the same in RA and control patients [93], whereas in one study serum IL-2 inhibitor was increased in RA, suggesting decreased IL-2 activity [94] and in another it was decreased [95]. These contradictory findings do not support an unequivocal role in vivo of IL-2 in ACD in RA.

IL-3 (multi-CSF) was shown to stimulate bone marrow cells to form myeloid colonies [96]. It potentiated the effects of G-CSF and GM-CSF in vitro. Erythroid colony growth was also stimulated by IL-3 addition to the marrow cultures [97]. It is not known whether this phenomenon might serve to counteract effects of other interleukins and prevent ACD in RA in vivo.

IL-6 is another monokine with biological activities related to inflammatory responses [98,99]. IL-6 levels were elevated in synovial fluid of patients with RA [100]. It is able to stimulate erythroid colony growth especially in the presence of IL-3 [101,102], although others found no effects on erythroid colony growth [103]. For its possible stimulatory effects on erythropoiesis it might play a counter regulatory role to other interleukins in ACD.

It was shown that tumor necrosis factor (TNF) was able to induce resorption of proteoglycan and to inhibit proteoglycan synthesis in cartilage in vitro, suggesting a pathogenetic role in RA [104]. Goodman [105] found that both activated macrophages and TNF, one of their products were able to suppress erythroid colony growth. Lu also showed inhibitory effects of TNFα on erythropoiesis [76]. In another study it was shown that TNFα addition resulted in release of multipotential colony stimulating activity from fibroblasts [106] through which it might possibly counteract its direct suppressive effects on erythropoiesis in vivo.

TABLE 3 Possible role of mediators of erythropoiesis in ACD and their effects on erythropoiesis

MEDIATORS OF	ERYTHROPOIESIS			ROLE IN
ERYTHROPOIESIS	INCREASE	DECREASE	NO EFFECT	ACD
ACIDIC ISOFERRITIN		73*,74*,75*		?
PROSTAGLANDIN	76,78		77	?
INTERFERON ALPHA	81			-
INTERFERON GAMMA		79,80		+/?
INTERLEUKIN-1		77,89		++/?
INTERLEUKIN-2		92	93	+/-
INTERLEUKIN-3	96**,97			?/?
INTERLEUKIN-6	101,102		103	-/?
TNF ALPHA	106	76,105		++/-

(- = positive observation/no role in ACD; + = positive/ role in ACD; ? = doubtful). Numbers reflect references cited.
* decrease in granulocyte and macrophage progenitor cells.
** stimulation of myeloid colonies.

All studies described above deal with in vitro effects of mediators of erythropoiesis. In the various studies cultures from various subsets of patients are examined, e.g., normals, patients with RA or leukemia. Results from these studies often are difficult to compare. The inhibitory effects of IL-1 and TNF on bone marrow precursors seem to be consistent. Although some biological effects of these cytokines have been described in the clinical situations it is not yet proven whether the inhibitory effects on erythropoiesis also are present in vivo. Elevated serum levels reflect disease activity but do not prove a role in ACD. We believe the best approach to prove

in vivo effects is to measure monocytic cytokine production from inflammatory joints and bone marrow before and after antirheumatic treatment and to correlate these differences with changes in Hb and erythroid colony counts. Another - theoretical - method would be to administer interleukins, prostaglandins or interferons, and antibodies to these substances to patients and follow erythropoiesis. The effects of antirheumatic treatment modalities on bone marrow cells are difficult to avoid but should be kept as constant as possible in both transversal and longitudinal studies.

1.4.6 Hemolysis and red cell life span

Many authors found a reduced red cell life span in ACD ranging from 81-90 days in anemic RA patients and 98-114 days in controls [68,107]. It is possibly explained by increased phagocytic capacity of macrophages [108] resulting in a higher clearance rate of erythrocytes [2,3,109].

Since fever may result in erythrocyte damage [110], especially in older ones, this might also contribute to a decreased red cell life span. Shortening of erythrocyte survival in these studies is only moderate and shows considerable overlap with controls, while fever is not very frequent in RA and is certainly not specific for anemic RA patients. The absence of an increased reticulocytosis as a reaction to hemolysis further points to other more important factors involved.

1.4.7 Erythropoietin

Generally in patients with normal renal function serum erythropoietin rises when anemia or a hypoxic stimulus is present [111,112]. Erythropoietin is reported to stimulate the rate limiting enzym in haemsynthesis, delta amino laevulinic acid synthetase (ALA-5). The rate of haemsynthesis was found to be lower in anemia of chronic infection [113]. Failure of ALA synthetase rise in response to anemia, pointing to a marrow defect or an inadequate erythropoietin response, was also suggested [114]. Some authors found erythropoietin levels to be lower than expected for the degree of anemia in RA [115,116]. Cotes, using a sensitive radioimmunoassay, found

erythropoietin levels within the normal range, which were independent of the type of anemia [117], although he also showed that some RA patients did have inadequate erythropoietin levels. The same controversy was present in anemia associated with malignancy and infection [118]. Zucker [119] suggested rather a decreased bone marrow responsiveness to erythropoietin than a decreased level in the case of malignancy. More recent studies are still controversial. Erslev [112,120] found higher levels of erythropoietin in anemic patients irrespective of the cause which is in agreement with a study by Birgegard [121], who found a correlation between hemoglobin, ESR and erythropoietin level. In RA patients it was suggested that disease activity dictates Hb level which in turn causes a rise in erythropoietin level. This is in contrast with Baer, who saw lower erythropoietin levels in anemic RA patients compared to other anemic patients [122]. Since in most studies a negative correlation is found between Hb and parameters of disease activity it could be that Epo production rises when RA activity declines resulting in a Hb increase.

From the above data we conclude that the role of a decreased Epo responsiveness in ACD is not unlikely in ACD but it could be a side phenomenon of increased RA disease activity. Its specific role can only be proven when a Hb rise follows administration of exogenous recombinant DNA erythropoietin while leaving antirheumatic treatment unchanged.

1.4.8 Erythroid progenitor cells

Since the discovery of methods for culturing erythroid progenitor cells in methylcellulose as a medium, much research has been carried out to establish a possible mechanism at stem cell level in the pathogenesis of ACD. Two types of erythroid cells can be cultured, colony forming units (CFUe) and burst forming units (BFUe). Growth of these cell lines generally are stimulated by T cells and macrophages [123]. It was found that CFUe growth in the presence of fungal infection [124] and cancer cells [119], was inhibited in experimental animals. The same inhibition occurred in mice after abcess induction by turpentine [125]. In normal mice CFUe growth was stimulated by erythropoietin, while in mice with an abcess erythropoietin was much

less able to stimulate growth.

In RA patients with and without anemia Reid found CFUe and BFUe growth was not different [126]. Depletion of macrophages resulted in decreased growth in both groups while readdition restored growth. Harvey et al. [127] found that mean BFUe colony number from peripheral blood of anemic RA patients was lower than nonanemic RA and control patients, but this was not significant. They also found decreased growth after T cell depletion in all groups. Prouse et al. [128] also reported stimulating effects of T cells on BFUe growth in both normal volunteers and anemic RA patients. Other authors in contrast reported that peripheral T cells from anemic RA patients inhibited CFUe growth in normal subjects [129]. This was not the case when T cells of normal subjects were used. Autologous serum inhibited BFUe growth in anemic RA patients as well as in nonanemic RA patients. In another study, however, only serum from 2 of 7 anemic RA patients inhibited erythropoiesis [129]. Serum from nonanemic RA patients stimulated BFUe growth [126]. Others found an inverse relationship between BFU colony numbers and serum IgM titer and rheumatoid factor [127].

Data from studies using in vitro experiments with erythroblasts should be interpreted cautiously. In these studies erythroblasts are cultured in media consisting of other marrow cells and serum. These factors might either stimulate or inhibit erythroid colony growth for instance by production of certain interleukins in the cultures. The findings can not be extrapolated to the clinical situation. The finding of a negative correlation of IgM rheumatoid factor level and BFUe count [127] therefore is coincidental in our opinion. A possible connection could be that bone marrow cultures of RA patients with ACD produce for instance more IL-1 or TNF than in those of nonanemic RA patients resulting in a reduction of erythroid colony growth (see under section: Mediators of erythropoiesis). So far, data lack to prove this assumption.

1.5 FUNCTIONAL ASPECTS OF ACD

In most of the above theories the development of ACD could be explained by a decreased erythropoiesis. A decreased iron availability

is one of them. The question arises what purpose is served by this phenomenon. In infection it is known that hypoferremia might be a protective factor because most micro-organisms need iron for their metabolism [130,131,132]. Lactoferrin and transferrin inhibit bacterial growth and this inhibition is decreased when iron is added to saturate these proteins [133]. Hershko [134] recently found in a review of studies on iron deficiency and infection beneficial effects of iron deficiency, although not always consistent. Andrews saw reduction in joint inflammation in Wistar rats when they were made iron deficient, although this was not seen in a change in serological parameters [135]. It is also stated that a high level of synovial iron anticipates poor prognosis [136] in RA and arthropathy can be a manifestation of iron overload without arthritic disease [137]. It is also claimed that iron treatment may be deleterious in RA patients because it might worsen the synovitis [138,139].

It may be concluded that hypoferremia plays a protective role in RA. The finding of a high prevalence of iron deficiency among RA patients [11], impaired iron absorption in active RA [41], a possibly diminished MPS iron release [47,48] and trapping of iron by ferritin and lactoferrin [53,63] might point to a mechanism which prevents iron deposition in joints to some extent. Whether the anemia in ACD as such has beneficial effects is not known.

1.6 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The suspicion of the presence of ACD rises when the patient has a chronic disease, reflected by clinical and laboratory signs, and anemia, when no other apparent causes of anemia are present. In the case of RA serological markers of disease activity are erythrocyte sedimentation rate, C-reactive protein, C1q binding assay and Rose titer. Correlation exists between disease activity and presence and severity of ACD [3,121]. If hypoferremia, decrease in iron saturation of transferrin capacity, normal to decreased serum transferrin are also present (Cartwright criteria; [1,2,3]) together with a relative low reticulocyte count the diagnosis seems probable. Generally the anemia is normochromic normocytic [1,2,3,4] but hypochromasia and

microcytosis may be present without concomitant iron deficiency [4], especially in juvenile rheumatoid arthritis [140].

The diagnosis of ACD is made by exclusion since many other causes may be present in RA. Gastrointestinal blood loss, adverse reactions of antirheumatic drugs, autoimmune phenomena and vitamin deficiencies, which are more frequent in RA than in a control population as was discussed previously, should be ruled out.

The main problem in differential diagnosis of ACD in RA is the presence of concomitant iron deficiency. Iron deficiency is reported in frequencies from 30 to 70% among anemic RA patients [7-12]. The most reliable parameter for the detection of iron deficiency is stainable iron content in a bone marrow aspirate [141,142]. No or scarce amounts of stainable iron point to iron deficiency while an increased amount of it fits the concept of ACD. Because of the high prevalence of anemia and iron deficiency in RA bone marrow aspiration is not a practical approach. Therefore, a number of studies were performed to correlate serological and erythrocyte parameters with stainable bone marrow iron content. Simple iron deficiency in patients without chronic disease is easily recognized by: microcytosis, hypochromasia, low serum iron, high transferrin, a low serum iron to transferrin ratio and a low serum ferritin [143,144]. In RA, if iron deficiency is present, it generally coexists with ACD. Although in ACD all these parameters can be similar to what is found in iron deficiency although transferrin generally is lower and ferritin higher [1,2,3,4] it was shown that MCV and MCHC can be reduced in 32 and 49% of patients respectively in ACD without iron deficiency [4]. In juvenile rheumatoid arthritis and anemia MCV can be as low as 60 fl without iron deficiency [140]. In other studies, however, MCV at a level of 80 fl turned out to be the most specific and predictive parameter for the detection of iron deficiency [11]. MCH is not a valid marker of iron deficiency [11]. Recent work shows that red blood cell distribution width (RDW) varies with MCV and Hb but no significant difference exists between ACD with and without iron deficiency [145]. RDW apparently correlates more with the severity than the type of anemia.

Serum iron in ACD does not correlate with body iron stores in anemic RA patients [11] and anemic geriatric patients [146].

Serum transferrin levels generally are lower in ACD and for the detection of iron deficiency it is not a valid test [11].

Serum ferritin, however, correlates best with stainable bone marrow iron content [11,146,147] in RA patients. Different cut off points (c.o.p) are used for the diagnosis of iron deficiency in RA. Patterson [146] showed that 45 μg/l was optimal, Hansen [11] and Rajapakse [9] used 60 μg/l. In healthy individuals lower limit is 12 μg/l.

Erythrocyte-ferritin was shown to be a reliable parameter for the detection of iron deficiency in patients without chronic disease [148] but in the evaluation of anemia in RA it was not very useful [149].

The usual findings of iron deficiency in peripheral blood are also present in anemic iron deficient RA patients. For its detection erythrocyte and serological parameters should be adapted because coexistent ACD tends to change these parameters in an opposite direction than iron deficiency does.

Age might be a contributive factor. Mean age is frequently above 60 years in RA patients [11] and it is known that in elderly people unexplained anemia is more common [150], possibly due to a defect in hematopoiesis in aging [151].

It is clear that the diagnosis of ACD can only be made by exclusion of other types of anemia. As was shown in other paragraphs other causes of anemia frequently coexist with ACD. Coexistent iron deficiency remains the most important differential problem. A bone marrow aspiration for iron status remains the golden standard. We conclude that in all anemic RA patients iron status should be examined to direct a diagnostic work-up of iron deficiency as well as serum B12 and folic acid whereas hemolysis and toxic effects of drugs should be ruled out as well.

1.7. TREATMENT

ACD generally is a mild and nonprogressive type of anemia resulting in a hemoglobin level rarely less than 70% of normal. Treatment, however, is only absolutely indicated when coexistent

angina pectoris, heart failure or respiratory failure is present. A major operation might also be an indication. Relative indications are pregnancy, reduction of exercise tolerance, fatigue, palpitations, dizziness, tinnitus and headache. Depending on severity of anemia and the underlying disease, it is to be decided whether blood transfusion or more longstanding therapy is needed. If other causes of anaemia are present they should be treated correspondingly. When antirheumatic drug toxicity is involved the drug must be withdrawn and the patient must be treated in a different way.

Since there is some correlation between RA disease activity and severity of anemia [3,121], while nonanemic RA patients usually have a lower disease activity compared to anemic RA patients [2,3], the mainstay of management of the anemia is to treat the RA. Samson [67] showed that anemia and ineffective erythropoiesis was reverted after treatment of the RA with gold. It was shown that 3 months of antirheumatic treatment in patients with RA and ACD resulted in a significant Hb rise and decrease in disease activity, independent of treatment modality [121].

In the past ACD was treated succesfully with cobalt [152] but because of its toxicity this is not suitable for longterm treatment.

A temporary rise in Hb and MCV may follow intravenous or intramuscular injection of high doses of iron [153,154]. After treatment Hb fell to initial values. Others reported that only anemic RA patients with iron deficiency respond to iron treatment [9,155]. If the clinical situation does not point to secondary causes of iron deficiency it is difficult to decide to treat anemic RA with iron without performing a bone marrow aspiration for assessment of stainable iron. As in the diagnosis of iron deficiency ferritin might be a predictor of iron responsiveness of the anemia [155]. RA disease activity declined during the course of iron treatment in these studies, which may be an important contributive factor in Hb rise. On the other hand it can be concluded that iron treatment in iron deficient RA patients is not harmful. Reddy [138] and Blake [139], however, found worsening of disease activity but they used high parenteral doses. As was discussed in a previous section iron deficiency, indepent of its cause, might be protective in RA [130,131].

Based on the observations of the possible deleterious effects of iron on synovitis some authors evaluated the effect of iron chelators. In animal models it was shown that desferrioxamine, an iron chelator, stimulated the acute inflammatory reaction, but inhibited chronic inflammation in RA [156]. Andrews [157] showed that soft tissue swelling and bone erosions decreased in Wistar rats using desferrioxamine in a dose of 100 mg/kg. In human RA patients a dose from 2 to 3 gram daily resulted in many toxic side effects, although in some of the patients inflammation improved [158,159]. Later when a dose of 1 gram daily was used no apparent side effects were found while pain, morning stiffness and ESR decreased [160]. In these studies a Hb increase was reported after two weeks of treatment, while a single dose of 1 gram resulted in a Hb rise already in 1 week [161]. In another study [162] it was shown that in 5 patients with RA treated with desferrioxamine for 6 months no improvement in RA activity was seen while Hb and ferritin decreased significantly. Recently it was shown that desferrioxamine treatment also raises Hb in hemodialysis patients after 3 months of treatment [163]. In all studies Hb declines after cessation of treatment. It is postulated that iron chelating agents increase transferrin receptor expression in human erythroblasts [164,165].

Since desferrioxamine can only be administered parenterally it is worthwhile to evaluate the effects of new oral chelating agents on ACD in RA. They have shown to be effective in thalassemia, myelodysplastic syndromes and transfusional hemosiderosis [166,167]. The use of iron chelators may have the advantage of both reducing disease activity [156,157] and to correct the anemia [161] but results are few and short term. In patients treated with desferrioxamine for 6 months, however, a significant Hb and ferritin decrease was found [162]. Baseline ferritin in these patients was low, however, suggesting iron deficiency instead of ACD. Long-term studies are necessary to evaluate the effect of oral iron chelation therapy on erythropoiesis, RA activity and potential toxicity.

Although the role of erythropoietin in the pathogenesis of ACD remains to be established, it will be interesting in both therapeutic and pathophysiologic point of view to treat anemic RA patients with erythropoietin. It was found earlier that hypoxic stimuli could

correct ACD in rats suggesting an erythropoietin effect [168,169]. Exogenenous recombinant DNA erythropoietin is effective in the treatment of anemia in end-stage renal insufficiency [170,171]. Recombinant technology will increase the availability of the hormone [172] and therefore other indications than renal insufficiency, like ACD, have to be investigated.

1.8 DISCUSSION AND SUMMARY

ACD is a frequent complication of chronic disease. Most of the studies considering diagnosis and pathogenesis of ACD have been carried out in RA patients because of the high prevalence in and homogeneity of this patient group. This, however, limits extrapolation of certain features in ACD to other chronic diseases (Table 1) since they could be connected to RA as such.

Within the RA group itself diversity exists as well. Many studies reviewed here lack proper definition considering their iron status. This may have an important impact on interpretation of results, especially in those studies examining altered iron metabolism in ACD. This is illustrated by the fact that features as decreased MPS iron release and ineffective iron turnover were originally thought to be specific for ACD, but later it was shown to be associated with iron deficiency as well. Nearly all these studies have been carried out transversally. It is therefore recommended in future studies considering iron absorption, MPS iron release and IIT that they will be performed in a longitudinal fashion. These factors should be examined before and after iron treatment in iron deficient RA patients. In ACD iron chelators should be administered before and after which the same parameters should be studied since the observed increased erythropoiesis after this way of treatment might be due to increased bone marrow iron availability through iron release from ferritin, lactoferrin and the MPS. In the third arm of such a study RA patients with either iron deficiency or ACD should be treated antirheumatically. The same model can be used to prove a role of decreased erythropoietin responsiveness, for these studies lack consistency as well. Serum erythropoietin and parameters of

erythropoiesis in RA and ACD should be measured before and after either antirheumatic or exogenous erythropoietin treatment. The performance of such studies demands great efforts of both patients and investigators, but it will definitely solve many controversies.

Another problem frequently seen in studies cited here is the extrapolation of in vitro findings to the in vivo situation. This holds especially true for studies examining erythroid progenitor cells and effects of mediators of erythropoiesis. This was discussed in the respective sections. A possible approach -in case of determining effects of LEM- is to administer substances as prostaglandins, interferons or interleukins and their antibodies or antagonists to patients. Apart from ethical aspects the problem of in vitro phenomena remains, considering studies on in vitro erythropoiesis, unless one wishes to rely on simple parameters such as Hb, Ht and reticulocytes. Another solution, culturing without serum or monocytes in order to prevent interleukin production with intrinsic effects on in vitro erythropoiesis is under investigation.

We reviewed studies on pathogenesis, diagnosis and treatment of ACD in RA. Many pathogenetical factors are not unlikely to play a part in ACD such as impaired MPS iron release, iron trapping by iron binding proteins (such as ferritin and lactoferrin), decreased erythropoietin responsiveness to the anemia, suppressive effects on erythropoiesis of interleukins especially IL-1 and TNFα, although data from the studies cited are far from consistent. Factors as hemolysis and decreased red cell life span are of minor importance. Hypoferremia in ACD might play a protective role in RA since iron could worsen symptoms. Longitudinal studies might substantiate the value of the different pathogenetical factors but they are very hard to perform for practical reasons. Another problem remains the insufficient description of iron status in many studies making interpretation difficult.

The diagnosis of ACD depends on exclusion and the main differential problem is the detection of coexistent iron deficiency. Treatment, rarely indicated, should be directed towards antirheumatic treatment. Specific treatment modalities such as (oral) iron chelators and exogenous erythropoietin have to be investigated for both pathogenetical and therapeutic reasons.

1.9 AIMS OF THE STUDIES

1.9.1 To assess the frequency of iron, vitamin B12 and folic acid deficiency in RA patients and to examine the effects of these deficiencies on certain diagnostic parameters in blood in comparison with non-anemic RA patients (Chapter 3).

1.9.2 To characterize the difference between ACD and ACD complicated by iron deficiency in RA by means of simple erythrocyte and serological parameters in order to predict the presence of iron deficiency by these parameters without performing a bone marrow aspiration for stainable iron.

1.9.3 To predict iron responsiveness of anemia in RA by means of simple erythrocyte and serological parameters and to evaluate whether iron treatment results in a serological increase of RA disease activity (Chapter 5).

1.9.4 To examine whether iron uptake by and transferrin binding to erythroblasts is defective in RA patients with ACD (Chapter 6).

1.9.5 To establish a potential pathogenetic role of a decreased erythropoietin responsiveness to anemia in RA patients with ACD and to examine whether Epo responsiveness is higher among iron deficient RA patients (Chapter 7 part one and two).

1.9.6 To evaluate whether iron release from iron stores by oral iron chelation in RA patients with ACD results in increased erythopoiesis and increased Epo responsiveness to anemia (Chapter 8).

1.9.7 To determine a possible potential role of TNFα in determining ACD in RA patients (Chapter 9).

1.9.8 To investigate whether IL-6 is involved in ACD pathogenesis (Chapter 10).

CHAPTER 2

PATIENTS, MATERIALS AND METHODS

2.1 PATIENTS

All RA patients included fitted the revised criteria of the American Rheumatism Association (ARA) and gave informed consent before entering the study. All protocols have been accepted by the Medical Ethical Committee of the Department of Rheumatology of the Dr Daniel den Hoed Clinic.

In some studies in this thesis overlap in patients occurred. Patients without B12 or folic acid deficiency from the study described in Chapter 3 were used in Chapter 4 and 5 as well. For the in vitro studies on erythroblasts (Chapter 6, 9 and 10) bone marrow from 39 subjects were used. The bone marrows used in Chapter 6 and 10 consisted of subdivisions of those described in Chapter 9. In Chapter 7 part one the results were obtained from a subset of patients described in Chapter 3. In Chapter 7 part two and Chapter 8 part of the patients were also used in Chapter 6, 9 and 10, whereas a number of other subsets were also included.

2.2 ERYTHROCYTE PARAMETERS (reference values)

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

Reticulocyte count was assessed by vital staining with brilliant cresyl blue, expressed as a ratio of red cells ($^{0}/00$).

Mean corpuscular volume was calculated from hematocrite and number of erythrocytes ($^{Ht}/$ery's). (80-96 fl).

Mean corpuscular hemoglobin was calculated from hemoglobin and number of erythrocytes ($^{Hb}/$ery's). (1600-2200 amol).

2.3 PARAMETERS OF IRON STATUS (reference values)

Serum iron was assessed by colorimetric analysis of a ferrous iron/ferene complex at 595 nm without deproteinization and with reduction by ascorbic acid (Instruchemie, Hilversum, The Netherlands). (14-30 μmol/l).

Transferrin was determined with a Baker nephelometer 420 (Ablon Medical Systems, Leusden, The Netherlands). (44-80 μmol/l).

Serum ferritin was measured by solid phase enzyme immunoassay (Ferrizyme, Abott Labs Chicago, USA). (20-150 μg/l).

Erythrocyte-ferritin was assessed after centrifuging erythrocytes with removing of the buffy coat and supernatant. The sedimentated erythrocytes were resuspended with 166 mM NaCl. This washing procedure was repeated twice. The final red cell sediment was hemolyzed at 0° C after which the supernatant was tested for ferritin [1].

2.4 VITAMIN B12 AND FOLIC ACID ASSAY

Vitamin B12 and folic acid were measured using a radioassay technique (Dualcount, Diagnostic Products Corp., Los Angeles, USA). Reference values vitamin B12: > 150 pmol/l and folic acid > 7 nmol/l).

2.5 PARAMETERS OF DISEASE ACTIVITY (reference values)

Erythrocyte sedimentation rate was determined using the Westergren method (< 10 mm/h).

C-reactive protein was measured by using radial immunodiffusion partigen plates (Behring Werke, Marburg, West-Germany) (< 6 mg/l).

Clq binding assay was measured by a method originally described by Zubler et al. [2] (< 7%).

Waaler Rose test was assessed using sensitized sheep erythrocytes; a titre more than 1/32 was considered positive [3].

2.6 *GROWTH FACTORS*

2.6.1 *Erythropoietin*

Erythropoietin in serum was assessed using a sandwich radioimmunoassay with monoclonal antibodies. In 30 healthy donors mean Epo was 14.5 $\pm$ 4 U/l which was considered normal [4]. In the other assay a sandwich assay with a double antibody assay was used [5]. In 100 control sera mean Epo level was 22 $\pm$ 4 U/ml).

2.6.2 *Tumor necrosis factor*

Serum TNFα was measured immunoradiometrically using monoclonal antibodies against distinct epitopes of TNFα after coated tube separation. (s.a. IRE-Medgenix, Fleurus, Belgium) (reference value: 6.3 pg/ml, range 5-8).

2.6.3 *Interleukin-6*

IL-6 in serum was measured in the B9 assay [6]. Serum was heated for 30 minutes at 56°C and a titration was added to 5000 B9 cells and compared with standard IL-6 preparation. After 3 days proliferation was measured by thymidine incorporation. 1 U/ml is a concentration that leads to half maximal proliferation. In 100 controls serum levels were less than 10 U/ml.

2.7 *URINE IRON EXCRETION*

This was assessed using atomic absorption spectophotometry.

2.8 *IRON ABSORPTION TEST*

Serum iron was measured after an overnight fast after which a tablet containing 105 mg elementary iron (Ferogradumet^{R}, Abbott BV,

Amstelveen, The Netherlands) was ingested. After 2 hours serum iron was measured again.

The increase in serum iron expressed as a ratio of initial value was used as a parameter of iron absorption [7].

2.9 *BONE MARROW ASPIRATION*

Bone marrow was aspirated after sternal or posterior iliac crest puncture.

2.9.1 *Stainable iron content*

Iron content was measured by staining with Perl's Prussian blue. A semiquantitative scale was used. 0 = no stainable iron, 0 - 1 = minimal to very small amount, 2 = slight small and patchy content and 3 or more = increased stainable iron. On this scale patients with a stainable iron content of 0-1 were considered iron deficient [8,9].

2.9.2 *Preparation of cell suspension*

Twenty ml bone marrow aspirate was 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). After centrifugation the mononuclear cells were harvested, washed twice in HBSS and resuspended in HBSS [10].

2.9.3 *BFUe assay*

A cell suspension of 1.10^5 cells was added to a mixture of Iscove's modified Dulbecco's medium (IMDM), 0.40 ml with 2% methylcellulose, 0.30 ml fetal calf serum, 0.1 ml mixture (containing BSA, transferrin, lecithin, sodium selenite and 2-mercaptoethanol) after which 0.015 ml erythropoietin (1 U/l) was added. This volume was divided over 4 petridishes which were incubated at 37°C and 100% humidity in an environment of 5% CO_2 in air. Burst forming units (BFUe; containing 50 or more cells) were counted after 14 days (range 13-15) [10].

2.10 IRON INCORPORATION INTO AND TRANSFERRIN BINDING TO ERYTHROBLASTS

Iron incorporation into and transferrin binding to erythroblasts were measured as follows. Human transferrin (Behring Werke, Marburg, West Germany) was labelled with ^{125}I (Amersham Radiochemical Centre, UK) using Iodogen reagent (Pierce Chemicals, Rockford, IL) as catalyst. Another solution, containing a mixture of I_2, NaI and $Na^{125}I$, was added slowly to the transferrin solution at 0°C. After 5 hours of mixing at 4°C the surplus of ^{125}I was separated by anion exchanging (Amberlite IR Cl^-) [11]. ^{59}Fe(III) chloride was added to the purified transferrin solution until 100% saturation [12]. A sodiumbicarbonate solution (5%) was also added as an anion-donor which is necessary for Fe(III) binding to transferrin [13,14].

The cells were suspended in 10 ml of DMEM containing 0.5% BSA solution with addition of DNAse (30 mg/100 ml). Five nanomol transferrin was incubated per 15.10^6 nucleated cells for 2 hours at 37°C. After 2, 30, 60 and 120 minutes duplicate samples of 0.5 ml were taken and stored in cold PBS. The samples were centrifugated at 1200 rpm and 4°C during 7 minutes after which the supernatant was removed. Cells were then washed in PBS and centrifugated twice again after which activity of the cells was measured using a Packard-autogamma 500.C. (13,14). I^{125} counts were corrected afterwards for ^{59}Fe spill-over (13% of ^{59}Fe counts). In all experiments non-specific cell bound radioactivity -usually less than 1 pmol/10^6 cells- was subtracted from the obtained experimental values.

2.11 STATISTICS [15]

2.11.1 Student-t-test

This test was used for analysis of normally distributed data. The following formula was used:

$$\text{I} \qquad S = \left[\frac{(N_1-1).S_1{}^2 + (N_2-1).S_2{}^2}{N_1 + N_1 - 2} \right]^{\frac{1}{2}}$$

II $$X_{1(mean)} - X_{2(mean)} \pm t½\ \alpha.S. \left[\frac{N_1 + N_2}{N_1 + N_2} \right]^{½}$$

S = test value
$N_1 N_2$ = number of observations in population 1 and 2
$S_1 S_2$ = standard deviation population 1 and 2
$X_1 X_2$ = mean of observations in population 1 and 2
The result of formula II should be more than zero.

2.11.2 *Mann Withney U test*

This test was used for analysis of non-parametric data. Observations of both populations were ordered from low to high. Each observation was given a ranknumber. The following formula was used:

$$Sx_{minimal} = \frac{M(M+1)}{2} \quad Sx_{maximal} = \frac{M(M+2N+1)}{2}$$

M = sum of ranknumbers in population 1
N = sum of ranknumbers in population 2
Sx_1 = test value for population 1
If Sx is much larger or smaller than M(M+N+1)/2 it is concluded that the populations are different.

2.11.3 *Spearman's rank correlation test*

This test was used for correlation of data. Paired observations are given ranknumbers. The following formula was used:

$$Rs = 1 - \frac{6\ \Sigma\ V_1^{\ 2}}{N(N^2-1)}$$

$(V_1 = R_1 - S_1)$

$-1 \leq Rs \leq 1$

R_s = rank correlation coefficient
R_1 = ranknumber of observation 1^a
S_1 = ranknumber of observation 1^b
ΣV_1 = sum of differences in ranknumbers of paired observations (1^a and 1^B, 2^a and 2^b etc.)
N = number of paired observations

2.11.4 Wilcoxon sign-rank test

This test was used for comparison of data from the same patients before and after treatment.

2.11.5 Parameters of prediction of iron deficiency (chapter 4) and responsiveness of anemia to iron (chapter 5).

Sensitivity was calculated as the ratio of patients with iron deficiency, who are positive for the variable and specificity was calculated as the ratio of patients without iron deficiency who are negative for the variable. Validity reflected the sum of all patients with iron deficiency and a positive variable and patients without iron deficiency and a negative variable expressed as a ratio of all patients. Predictive value represented the ratio of the number of patients with a positive variable who were iron deficient (see schedule below).

	I + II* (iron deficient)	III + IV* (not iron deficient)
positive variable	A*	C*
negative variable	B*	D*
sensitivity = A/A+B specificity = D/D+C		validity = A+D/A+B+C+D predictive value = A/A+C

* number of patients

In the same way these parameters were used to predict a Hb rise in anemic RA patients who were treated with iron.

	Hb rise	Hb unchanged or decreased
positive variable	A*	C*
negative variable	B*	D*
sensitivity = A/A + B specificity = D/D + C	validity= A + D/A + B + C + D predictive value = A/A + C	

* number of patients

CHAPTER 3

DIAGNOSIS OF ANEMIA IN RA. THE INFLUENCE OF IRON, VITAMIN B12 AND FOLIC ACID DEFICIENCY ON BLOOD PARAMETERS IN PATIENTS WITH ACD

3.1 INTRODUCTION

In patients with active RA anemia is frequently present [1,2]. Many types of anemia are associated with active RA. Vitamin B12 and folic acid deficiency are reported to be more prevalent among RA patients than controls [3,4]. Iron deficiency is found in a high frequency in RA [5,6]. Many RA patients with active disease also have ACD, originally described by Cartwright [7].

In patients with active RA more causes of anemia, associated with RA, may be present simultaneously. Studies on diagnosis and pathogenesis may thus be difficult to interpret.

We therefore performed a study to assess the frequency of iron, vitamin B12, folic acid deficiency in RA patients and to examine the effects of these deficiencies on certain diagnostic parameters in blood in comparison with nonanemic RA patients.

3.2 PATIENTS AND METHODS

3.2.1 Patients

Thirty-six patients (5 males) with definite or classical rheumatoid arthritis were studied. Patients who were recently treated with iron, folic acid or vitamin B12 were excluded. Patients with a present or past ulcer history, hypermenorrhoe, hematuria, positive stools for occult blood, hemolysis or decreased creatinin clearance were also excluded. Overall disease duration was 7 years, 62% of the patients used longacting antirheumatic drugs while 82% was treated with nonsteroid anti-inflammatory drugs. Patients were divided into 3 groups. Group I (n=11) consisted of RA patients without anemia. Group II (n=13) consisted of anemic and iron deficient RA patients and group III (n=12) consisted of anemic patients without iron deficiency; they

were considered as having ACD (grade 2 or more stainable bone marrow iron, see Chapter 2.9.1). In group I mean age was 54 years, in II 62 and in III 65 years. The difference between I and III was not significant ($p<0.20$). No difference in disease duration and antirheumatic drugs used were found in three groups.

3.2.2 Laboratory procedures

The following parameters, described in Chapter 2, were assessed:

Erythrocyte parameters:
Hb, MCV, MCH, reticulocyte count.
Iron status:
serum iron, transferrin, ferritin.
Vitamin B12, folic acid.
Parameters of disease activity:
ESR, CRP, Clqba and Rose titre.
Bone marrow was aspirated after sternal puncture in the anemic patients, after which stainable iron was determined.

3.3 RESULTS

3.3.1 Relationship between cellular indices, stainable iron content, bone marrow iron content, serum vitamin B12 and serum folic acid

Iron deficiency occurred in 13/25 (52%) of anemic patients (Table 1).

MCV was higher in I compared to II ($p<0.002$) and III ($p<0.02$) while in III it was higher than in II ($p<0.05$).

MCH was higher in I compared to II ($p<0.001$) and III ($p<0.001$). It tended to be higher in III vs II ($p<0.20$).

Reticulocytes were lower in I vs II ($p<0.05$) and III ($p<0.02$). The difference between II and III was not significant.

Serum B12 was lower in group III compared to I and II but this was not significant (Table 1). In Table 2 it is shown that B12 deficiency occurred most frequently in group III but differences were not significant.

TABLE 1 Erythrocyte parameters, iron status, serum B12, serum folic acid and parameters of disease activity in group I (non-anemics), II (iron deficient patients) and III (ACD patients). Data are expressed as median with range.

	I (n=11)	II (n=13)	III (n=12)
Erythrocyte parameters			
Hb[1] (mmol/l)	8.1(7.6–8.4)	6.0(4.2–6.7)	6.3(5.4–7.1)
MCV (fl)	91(80–97)	78(71–100)	85(80–98)
MCH (amol)	1887(1630–2070)	1550(1310–2000)	1647(1520–1950)
reticulocytes (⁰/00)	10(1–23)	23(8–44)	32(13–69)
Iron status			
s-iron µmol/l	9(6–12)	4(1–8)	5(1–10)
transferrin (µmol/l)	65(52–79)	60(39–81)	51(39–65)
ferritin (µg/l)	31(10–190)	26(10–98)	90(45–221)
B12 and folic acid			
B12 (pmol/l)	213(117–557)	207(106–415)	165(38–319)
folic acid (nmol/l)	13.9(7.6–26.80	9.3(2.9–15.2)	9.0(5.0–13.9)
Disease activity			
ESR mm/h	36(14–60)	64(17–98)	90(49–128)
CRP (mg/l)	8(2–38)	26(2–117)	36(6–122)
Clqba (%)	5(3–39)	13(4–69)	29(7–100)
Rose titer[3]	96(32–512)	256(64–512)	96(32–1024)
(% positive)	(54%)	(62%)	(83%)

1) SI conversion: Hb in mmol/l x 1.61 = g/dl.
2) Rise in serum iron expressed as a ratio from initial value, see under "laboratory procedures".
3) Reciprocal titer of Rose test, patients with titers > 1/32 were considered positive.

*TABLE 2 Effect of low serum B12 (< 150 pmol/l) and low serum folic acid (< 7 nmol/l) on cellular indices in non-anemic patients (I), iron deficient patients (II) and ACD patients (III).**

	Serum B12		Serum folic acid	
	low (<150 fmol/l)	normal (>150 fmol/l)	low (<7 nmol/l)	normal (≥7 fmol/l)
I				
Number (%)	3(30%)	7	0	11
Hb (mmol/l)	8.1	7.9		8.1
	(7.7–8.4)	(7.6–8.3)		(7.6–8.4)
MCV (fl)	93	90		91
	(90–97)	(80–97)		(80–97)
MCH (amol)	1973	1839		1887
	(1860–2150)	(1630–2010)		(1630–2070)
II				
Number (%)	3(23%)	10	4(31%)	9
Hb (mmol/l)	6.5	5.9	6.4	5.9
	(6.4–6.6)	(4.2–6.7)	(5.8–6.7)	(4.2–6.7)
MCV (fl)	88	75[3)]	81	77
	(82–100)	(60–87)	(78–87)	(60–100)
MCH (amol)	1743	1491[3)]	1560	1544
	(1590–2000)	(1310–1730)	(1490–1640)	(1310–2000)
III				
Number (%)	4(36%)	7	1(9%)	10
Hb (mml/l)	6.5	6.3	6.6	6.4
	(5.7–7.1)	(5.9–7.0)		(5.4–6.7)
MCV (fl)	90	83[1)]	83	85
	(80–98)	(80–90)		(80–98)
MCH (amol)	1757	1596[2)]	1590	1651
	(1560–1950)	(1510–1780)		(1510–1950)

* In some patients serum B12 or folic acid was not assessed.
Data expressed as median and range [1)] $p<0.10$ [2)] $p<0.05$ [3)] $p<0.02$.

B12 deficiency was present in 10 patients, 8 of them (80%) had a normal Shilling test, 1 (10%) had gastric antibodies and 1 (10%) had undergone a gastrectomy.

Hb was slightly lower in patients without B12 deficiency in group II ($p<0.20$). MCV was higher in patients with B12 deficiency compared to patients without B12 deficiency in group I (ns) II ($p<0.02$) and III ($p<0.10$). MCH was higher in B12 deficient patients in group I ($p<0.20$) II ($p<0.02$) and III ($p<0.05$). No correlation existed between serum B12 level and MCV and MCH except for B12 and MCH in the anemic patients ($r=-0.36$, $p<0.05$).

Megaloblastic changes in bone marrow were present in 4 of 10 B12 deficient patients (40%).

Folic acid deficiency did not occur in the group of nonanemic patients (I). In group II it was found in 31% and in group III in 9% of patients (Table 2). No specific causes of folic acid were found. Table 1 shows that serum folic acid was lower in groups II and III compared to group I ($p<0.02$ for both). MCV and MCH were both higher in patients with folic acid deficiency in groups II and III but this was not significant. Megaloblastic changes in bone marrow were found in 1 patient (2%). In patients with normal B12 a low folic acid occurred twice while in patients with normal folic acid a low B12 was found in 3 patients. Folic acid correlated negatively with ESR ($r=-0.49$, $p<0.005$) and CRP ($r=-0.43$, $p<0.01$).

One patient had a combination of iron, B12 and folic acid deficiency (Hb 6.6, MCV 83 and MCH 1590) while another had ACD with folic and B12 deficiency (Hb 5.7, MCV 87, MCH 1730). After correction of these findings the differences in cellular indices were slightly larger but significance level did not change.

3.3.2 *Relationship between stainable bone marrow iron content and parameters of iron status*

Serum iron was below normal (14 μmol/l) in all RA patients. It was lower in groups II and III compared to group I ($p<0.002$ for both) (Table 1). Difference between groups II and III was not significant.

Transferrin did not differ in group I compared to II. It was lower in III compared to I ($p<0.01$) and II ($p<0.05$).

Ferritin was lower in group II compared to group I (ns). It was higher in group III vs I ($p<0.02$) and II ($p<0.002$). A correlation was found between ferritin and ESR ($r=0.49$, $p<0.05$) as well as CRP ($r=0.82$, $p<0.005$ in group II and $r=0.54$, $p<0.05$ in III while in group I no correlation existed between ferritin and CRP).

3.3.3 Parameters of disease activity (Table 1)

ESR was lower in group I compared to II and III ($p<0.002$ for both). It was higher in III compared to II but this was not significant.

CRP was lower in I than II ($p<0.10$) and III ($p<0.02$). The difference between II and III was not significant.

Clqba was lower in I compared to II ($p<0.10$) and III ($p<0.02$). The difference between II and III was not significant.

Rose titer was higher in II vs I ($p<0.10$).

ESR correlated with Hb only in the ACD group ($r=-0.52$, $p<0.05$). CRP and Clqba did not correlate with Hb.

3.4 DISCUSSION AND SUMMARY

In this study we evaluated the importance of deficiencies of iron, B12 and folic acid in the differential diagnosis of anemia in RA. It was shown that iron deficiency occurred in 52% of anemic patients based on stainable iron content in bone marrow. In the nonanemic patients iron deficiency was not ruled out since no marrow aspiration was performed in this group. ACD (normal to increased stainable bone marrow iron) occurred in 48% of the anemic patients.

Vitamin B12 deficiency occurred in about 30% of patients in all 3 groups (serum B12 lower than 150 pmol/l) and folic acid deficiency (serum folic acid < 7 nmol/l) was present in 21% of anemics (31% in group II and 9% in group III).

MCV and MCH were found to be lower in the iron deficient patients compared to nonanemic patients. A hypochromic microcytic anemia is frequently found in iron deficient patients without chronic disease [9]. In our study MCV and MCH were also lower in anemic RA patients

without iron deficiency (considered as ACD patients) although they were higher than in iron deficient patients. Other studies also reported a lower MCV and MCH (2,7,10) in ACD patients. Anemia caused by iron deficiency is therefore not easily distinguished from ACD in patients with active RA by means of MCV and MCH.

Reticulocytes were found to be higher in the anemic groups suggesting some response to the anemia. They tended to be higher in ACD but this was not significant. No difference in reticulocyte count was found between B12 and folic acid deficiency and the other types of anemia.

In evaluating differences in cellular indices considering the dichotomy iron deficiency versus ACD it should be realised that these differences may be a little masked because of the coexistent presence of B12 or folic acid deficiency. B12 deficiency prevalence was the same in all three groups while folic acid deficiency was present more often in iron deficient patients. B12 deficiency had an important influence on cellular indices most pronounced in iron deficient patients. Patients with a combination of iron and B12 deficiency had a normochromic normocytic anemia. So if one relies on cellular indices many patients would have been classified as having ACD in which normal cellular indices are frequently found [6,7]. B12 deficiency usually is associated with a hyperchromic macrocytic anemia [11] although B12 deficiency can occur in patients with normal cellular indices [12]. Iron deficiency is associated with a hypochromic microcytic anemia [9] so the combination results in normal indices. Folic acid deficiency exerted the same effects on cellular indices but these were not significant.

Serum iron was below normal in all patients including the nonanemic patients although it was lower in both anemic groups. It is known that serum iron does not distinguish between iron deficiency and ACD [5,7] which implies that it does not correlate with body iron stores. The low serum iron in nonanemics could be explained by the fact that in chronic disease, to some extent also present in this group, serum iron is low, possibly through trapping by ferritin [13] or the MPS [14,15].

The cause of the high prevalence of B12 deficiency among RA patients remains obscure. We found normal Schilling tests in most

patients and only one had gastric antibodies. Couchman [3] also reported normal Schilling tests in RA patients. Although significant effects upon MCV and MCH were shown, its relation with the anemia here is uncertain since B12 deficiency occurred in the same rate in nonanemics. Folic acid deficiency in contrast occurred predominantly among iron deficient RA patients suggesting malabsorption or dietary causes, which was not established in this study. The negative correlation of serum folic acid with RA disease activity might point to a role in ACD pathogenesis although it could also be argued that folic acid is utilized by proliferating synovial cells. For both B12 and folic acid deficiency suppletion of the deficieny followed by Hb increase would prove some role in the anemia. Further studies are therefore necessary. In this thesis we only focused on effects of these deficiencies on diagnostic parameters while iron metabolism was further investigated in ACD (Chapter 4, 5, 6 and 8).

It was shown that in most RA patients more causes of anemia were present simultaneously. We assume that iron deficient patients also have features of ACD since a negative correlation was found between Hb and disease activity (ESR) in the ACD group and parameters of disease activity were higher in iron deficient patients compared to nonanemics. This assumption was confirmed by the findings of a lower transferrin in iron deficient patients compared to nonanemics while ferritin was not low in both nonanemic and iron deficient patients while transferrin was found to be lowest and ferritin highest in ACD patients. Ferritin behaves like an acute phase reactant since it correlated with ESR and CRP. Among ACD patients iron deficiency can be detected most easily by means of a lower ferritin, although it is not subnormal [5]. The dichotomy ACD versus iron deficiency therefore is rather artificial. So, in fact we found that only 6 of 25 (24%) anemic patients had just one cause of anemia (ACD).

All other patients had combinations of iron, B12 or folic acid deficiency, with or without ACD. In 2 patients (8%) both folic acid and B12 deficiency occurred. This implies not only that accurate diagnostic attention should be paid to the approach of anemic RA patients but also that several therapeutic possibilities in the treatment of anemia in RA are present apart from treating the RA.

In summary, we investigated the influence of iron, B12 and folic acid deficiency on diagnostic blood parameters of anemia in patients with ACD. All deficiencies were found to be highly prevalent in anemic RA patients. Only 24% of patients had just one type of anemia (ACD). Cellular indices were unreliable in the differential diagnosis since iron deficiency was marked by B12 and to a lesser extent by folic acid deficiency while MCV and MCH were lower in both iron deficiency and ACD. Ferritin is useful in the detection of iron deficiency among ACD patients. These findings suggest that studies on pathogenesis of anemia in RA should be interpreted with caution since usually more than one cause of anemia is present. Because deficiencies are present frequently several therapeutical possibilities should be considered apart from treating the RA.

CHAPTER 4

DIAGNOSTIC SIGNIFICANCE OF ERYTHROCYTE AND SEROLOGICAL PARAMETERS IN THE DETECTION OF IRON DEFICIENCY AMONG RA PATIENTS WITH ACD

4.1 INTRODUCTION

As was shown in Chapter 3 the most frequent causes of anemia in RA are anemia of chronic disease (ACD) and iron deficiency. Others found frequencies of iron deficiency up to 70% in RA [1,2]. We found that, in case of coexistent B12 or folic acid deficiency, iron deficiency could be masked if one relies on cellular indices. It was also found that MCV and MCH were lower in both ACD and iron deficiency compared to nonanemic RA patients (Chapter 3). Therefore detection of iron deficiency among RA patients with ACD remains an important diagnostic problem. Stainable iron content in a bone marrow aspirate is a reliable parameter for detecting iron deficiency [3,4], but this is not a useful procedure considering the high prevalence of anemia in RA. Serum ferritin is a valid marker of iron deficiency in otherwise healthy patients [5], but it is less accurate in RA patients [1,2,6] because it behaves as an acute phase reactant [7]. Other parameters like transferrin and MCV correlate to some extent with stainable iron in bone marrow [2,8,9] while erythrocyte ferritin is reported to correlate with body iron stores as well [5,10].

We therefore performed a study to characterize the difference between ACD and ACD complicated by iron deficiency in RA, by means of simple erythrocyte and serological parameters, in order to predict the presence of iron deficiency by these parameters.

4.2 PATIENTS AND METHODS

4.2.1 Demographic features

Forty-four patients (9 male) with definite or classical rheumatoid arthritis and a serum hemoglobin (Hb) of less than 7.4 mmol/l and who did not receive iron treatment for at least six weeks

previously, entered the study after giving written informed consent. Other causes of anemia were excluded by history (present or past ulcer disease, other gastrointestinal disease or complaints, menorrhagia and hematuria), negative stools for occult blood, a normal vitamin B12, folic acid, coombs test, creatinin clearance and absence of microscopic hematuria.

Patients were classified according to stainable bone marrow iron content. Group I (no stainable iron), group II (0-1; small amount), group III (1-2) and group IV (3 or more) (Chapter 2.9.1.). Groups I and II were considered iron deficient [3,4]. The other classification was based on cellular indices (range for MCV 80-96 fl and for MCH 1600-2200 amol). Patients in group A were hypochromic microcytic, in B hypochromic normocytic, in C normochromic normocytic, in D normochromic microcytic and in E hyperchromic macrocytic. Mean age was 63.4 years. A mean disease duration of 4.9 years was found, while 79% of patients were seropositive (Rose test) with a mean titer of 221 (reciprocally). Sixty-nine percent of the patients received long-acting antirheumatic drugs and 78% used NSAIDs. These data, including age and sex did not differ significantly in the various subgroups.

4.2.2. *Laboratory procedures* (for description see Chapter 2)

Erythrocyte parameters:

Hb, Ht, reticulocyte count, MCV, MCH.

Iron status:

Fe, transferrin, ferritin, ery-ferritin, iron absorption test and staining of a bone marrow aspirate for iron.

Disease activity:

ESR, CRP, Clqba.

4.2.3 *Statistics*

Sensitivity, specificity, validity and predictive value were calculated as described in 2.11.5.

4.3 RESULTS

4.3.1 Relationship between stainable bone marrow iron content and erythrocyte parameters

Iron deficiency (group I and II) was found in 24 patients (55%) (Table 1). Hb level did not differ in the four groups.

MCV was smaller than 80 fl in 13 patients (54%) in groups I and II and in 2 patients (10%) in groups III and IV. MCV tended to be higher in group III but this was not significant.

MCH was smaller than 1600 amol in 17 patients (71%) in groups I and II and in 7 (35%) in groups III and IV. MCH was higher in III compared to II ($p<0.05$). Reticulocytes were higher in IV compared to II ($p<0.10$).

In Table 2 it is shown that hypochromic microcytic pattern was seen in 30% of the patients. Twelve of them (92%) were iron deficient. Hypochromic normocytic anemia occurred in 25%. Five of these (46%) were iron deficient. The most frequent type of anemia was normochromic and normocytic (39%). This was associated with an iron deficiency rate of 29% (5 patients).

In the iron deficient groups 13 patients (54%) had a hypochromic microcytic anemia, 5 patients (21%) were classified as hypochromic normocytic and 1 (4%) as normochromic microcytic.

In patients without iron deficiency (groups III and IV) 12 (60%) had a normochromic normocytic anemia, 6 (30%) were hypochromic normocytic, 1 (5%) hyperchromic macrocytic and 1 (5%) normochromic microcytic.

Hb was significantly lower in hypochromic microcytic and hypochromic normocytic patients compared to normochromic normocytic patients ($p<0.0025$) (Table 2), while Hb was also lower in the 2 patients with normochromic microcytic anemia (ns).

4.3.2 Correlation between stainable bone marrow content and erythrocyte and serological parameters

S-iron did not differ between groups I-IV (Table 1) and groups A, B and C (Table 2). S-iron was higher in D and E but this was not significant.

TABLE 1 Correlation of stainable bone marrow iron content (I=0, II=0-1, III=1-2, IV=≥3) with erythocyte and serological parameters (values expressed as median and range).

	I		II		III		IV	
	n=12 (27%)		n=12 (27%)		n=12 (27%)		n=8 (19%)	
Erythrocyte parameters								
Hb (mmol/l)[1]	6.0	(4.2–7.2)	6.1	(4.2–6.7)	6.2	(5.2–7.1)	6.2	(5.4–7.0)
MCV (fl)	80	(60–100)	80	(60–107)	86	(75–93)	83	(75–90)
MCH (amol)	1568	(1310–1730)	1516	(1140–2080)	1729	(1510–2060)	1603	(1410–1780)
Reticulocytes (⁰/00)	21	(10–38)	24	(8–36)	25	(13–41)	32	(19–45)
Iron status								
S-iron (μmol/l)	5	(2–7)	4	(1–8)	5	(1–10)	5	(2–8)
Iron absorption[2]	4.5	(0.8–10.5)	1.6	(0.8–11.0)	2.1	(0.9–3.5)	1.4	(0.8–2.2)
Transferrin (μmol/l)	61	(33–81)	55	(33–85)	50	(39–60)	48	(34–65)
S-ferritin (μg/l)	17	(10–36)	30	(10–119)	99	(21–247)	109	(45–246)
Ery-ferritin (μg/l)	156	(96–1329)	97	(51–233)	128	(81–1863)	132	(28–930)
Disease activity								
ESR (mm/h)	75	(17–104)	74	(34–120)	85	(45–124)	91	(40–116)
CRP (mg/l)	30	(3–93)	37	(2–117)	30	(5–122)	56	(10–106)
Clqba (%)	18	(4–33)	20	(6–69)	24	(5–57)	51	(18–100)

1) Hb in mmol/l x 1.61 = g/dl.
2) Increase in serum iron expressed as a ratio (see Chapter 2.8).

TABLE 2 Relationship between cellular indices (normal values for MCV 80-96 fl and for MCH (1600-2200 amol), Hb and iron status (values expressed as median and range).

	hypochromic microcytic (A)		hypochromic normocytic (B)		normochromic normocytic (C)		normochromic microcytic (D)		hyperchromic macrocytic (E)
	n=13 (30%)		n=11 (25%)		n=17 (39%)		n=2 (4%)		n=1 (2%)
Hb (mmol/l)[1]	5.9	(4.2–6.9)	6.1	(5.7–6.6)	6.5	(5.4–7.1)	4.7	(4.2–5.2)	6.1
S-iron (μmol/l)	4	(1–7)	5	(2–7)	5	(1–10)	8	(2–14)	8
Iron absorption[2]	2.3	(0.8–11.0)	1.6	(0.8–4.5)	1.8	(0.8–3.8)	5.6	(2.6–8.5)	3.3
Transferrin (μmol/l)	56	(33–81)	57	(40–70)	51	(34–70)	58	(48–67)	33
S-ferritin (μg/l)	20	(10–246)	80	(13–119)	95	(12–247)	49	(10–87)	21

1) Hb in mmol/l x 1.61 = g/dl.
2) Increase in serum iron expressed as a ratio (see Chapter 2.8).

Iron absorption was higher in group I compared to group II, III and IV ($p<0.05$) but data in group II versus group III and IV were not different (Table 1). It correlated negatively with ESR ($r=-0.36$, $p<0.05$) and CRP ($r=-0.43$, $p<0.05$) in groups III and IV.

Transferrin decreased from group I to IV (Table 1), but the difference was only significant between I and IV ($p<0.05$). It did not differ in respect to the type of anemia (Table 2), although a negative correlation was found between transferrin and the parameters MCV ($r=-0.48$, $p<0.0005$) and MCH ($r=-0.40$, $p<0.0005$).

S-ferritin correlated with stainable bone marrow iron content (all differences between the groups I to IV were significant; p at least < 0.05); it was lowest in group I and highest in group IV (Table 1). When cellular indices were normal s-ferritin was higher than in hypochromic microcytic patients ($p<0.02$) (Table 2). In group B it was higher than in A ($p<0.10$). A negative correlation was found between s-ferritin and transferrin ($r=-0.45$, $p<0.0005$).

The e-ferritin was highest in group I, but this was not significant. It did not correlate with any of the other parameters.

ESR, CRP and Clqba tended to be higher in groups III and IV compared to I and II. In groups III and IV a negative correlation was found between Hb and ESR ($r=0.40, p<0.05$) but not with CRP and Clqba. Ferritin correlated with ESR ($r=0.34, p<0.0025$) and CRP ($r=0.38, p<0.01$).

4.3.3 Sensitivity, specificity, validity and predictive value of erythrocyte and serological parameters for the detection of iron deficiency (Table 3, Figure 1)

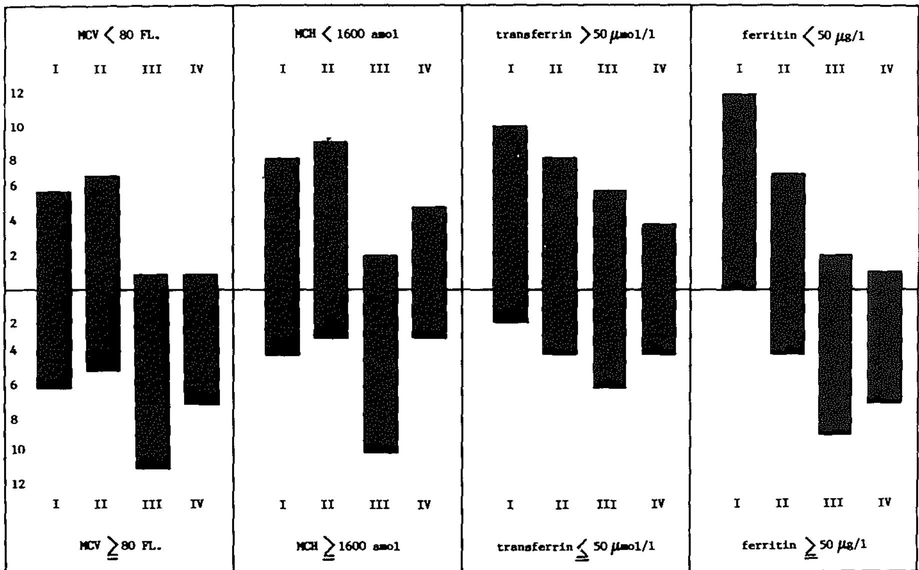
MCV (cut off point; c.o.p. 80 fl) has the highest specificity and predictive value for a single blood parameter. Its validity is low because of a low sensitivity. MCH (c.o.p. 1600 amol) has a little higher sensitivity but a lower specificity and predictive value. Combination of these parameters results in a higher, though still low validity, but this combination of parameters excluded 13 patients (30%) who had a combination of 1 parameter below and 1 parameter above its c.o.p. Transferrin (c.o.p. 50 μmol/l) has a low sensitivity and specificity and therefore a low validity. S-iron to transferrin ratio

does not increase the validity. The iron absorption test (not shown) had a low validity (51%). S-ferritin was the most valid single blood parameter (c.o.p. 50 μg/l). The combination of a MCV less than 80 fl and s-ferritin less than 50 μg/l yields a 100% specificity and predictive value (16 patients (36%) had a different combination). If patients also had a transferrin more than 50 μmol/l sensitivity became 100% as well (27 patients (61%) had a different combination).

TABLE 3 Sensitivity, specificity, validity and predictive value of erythrocyte and serological parameters and combinations for the detection of iron deficiency.

	Sensitivity %	Specificity %	Validity %	Predictive value %
MCV < 80 fl	52	90	71	87
MCH < 1600 amol	71	65	68	71
MCV < 80 fl MCH <1600 amol	72	92	77	93
transferrin > 50 μmol/l	75	50	63	64
iron/transferrin < 8%	65	65	64	68
ferritin < 50 /ug/l	82	84	83	86
ferritin < 50 μg/l MCV < 80 fl	79	100	89	100
ferritin < 50 μg/l transferrin > 50 μmol/l MCV < 80 fl	100	100	100	100

FIGURE 1 Relation between stainable bone marrow iron (I=0, II=0-1, III=1-2, IV=3 or more) and cellular and serological parameters. Horizontal midline is cut off point for the various parameters. Bars reflect the number of patients in relation to cut off points.



4.4 DISCUSSION AND SUMMARY

In this study we characterized the difference in erythrocyte and serological parameters between ACD with and ACD without iron deficiency and assessed the predictiveness of these parameters to detect iron deficiency. Stainable bone marrow iron content served as a standard for the diagnosis of iron deficiency [3,4].

Iron deficiency occurred in 55% of patients which is comparable to our finding of 52% (Chapter 3) and of other authors [1,2].

A reduced MCV was found in 34% of all patients but only in 10% of patients without iron deficiency. A low MCH was seen in 55% of patients and in 35% of patients without iron deficiency. Chernow [11]

found a reduced MCV in 32% and a reduced MCH in 49% of anemic RA patients with and without iron deficiency.

Hypochromic microcytic anemia was associated most (92%) and normochromic normocytic anemia least (29%) with iron deficiency. These types of anemia were present in iron deficient patients in 54 and 21%, respectively. This implies that the pattern of cellular indices most frequently found in iron deficient patients without chronic disease [12] also is found in patients with active RA. Patients with a hypochromic microcytic or hypochromic normocytic anemia had a lower Hb suggesting that iron deficiency in these patients was more pronounced resulting in lower cellular indices.

The majority of patients without iron deficiency, classified as having ACD, had either a normocytic normochromic (60%) or a normocytic hypochromic (30%) anemia. In ACD -in the absence of iron deficiency- a low MCH was frequently found (35%). The lower limit of MCH was changed from 1700 to 1600 amol in order to recognise concomitant iron deficiency because iron deficient patients also had a significantly lower MCH. MCV in ACD was generally higher though not significant and patients with iron deficiency generally may also have features of ACD since they have a certain disease activity. This would have masked a low MCV found in iron deficiency. Therefore lower limit of MCV was changed from 70 to 80 fl. If we had used normal ranges for MCV and MCH the anemias would have been classified a little different. It was shown before that, although MCV and MCH tended to be lower in both ACD and iron deficiency compared to nonanemic RA patients (Chapter 3), in iron deficiency these findings were much more pronounced.

The need of these adaptions was supported by the finding of an optimal sensitivity, specificity, predictive value and validity using these lower limits for MCV and MCH as cut off points for the detection of iron deficiency. Sensitivity of both MCV and MCH as well as their combination was low while specificity of MCV and predictive value were highest for a single erythrocyte parameter. The combination of a low MCV and MCH resulted in a higher specificity and predictive value. Sensitivity was also higher but remained low. So, the finding of a hypochromic microcytic anemia suggests that in RA ACD is complicated by iron deficiency and only a few patients would have been classified as having ACD while being iron deficient if no bone marrow aspiration

is done. Hansen et al. [1] found a specificity of 100% for MCV but sensitivity was correspondingly low (24%) which would -if no bone marrow aspiration is done- result in underdetection of iron deficiency. Of the two patients with normochromic microcytic anemia one was iron deficient while the hyperchromic macrocytic pattern found in another patient with ACD could not be explained.

No correlation existed between stainable bone marrow iron and serum iron, as we found earlier (Chapter 3), indicating that a low serum iron does not necessarily reflect low body iron stores.

Serum transferrin was found to be higher in iron deficient patients while it was inversely correlated with MCV MCH and s-ferritin, which is in agreement with the general findings in iron deficiency [12]. For obtaining optimal validity, using transferrin, which in our study was low in the evaluation of iron deficiency, the upper limit should be changed from 74 to 50 μmol/l because in ACD it is generally lower (Chapter 3).

A s-iron to transferrin ratio (iron saturation) less than 15% generally is diagnostic for iron deficiency in patients without chronic disease [12]. We found that a cut off point (c.o.p) of 8% was most useful but it provided no more information than s-transferrin alone in the detection of iron deficiency.

S-ferritin is a reliable parameter of iron deficiency in patients without chronic disease [5] but in chronic disease it behaves as an acute phase reactant [6,7], and therefore often a higher value is found, which may mask iron deficiency. In this study s-ferritin correlated with CRP and other parameters of disease activity like ESR and Clqba, confirming its acute phase behavior in active RA. Serum ferritin was highest in patients with a normochromic normocytic anemia. For the detection of concomitant iron deficiency lower limit of s-ferritin should be raised from 14 to 50 μg/l. At this level s-ferritin as a single parameter turned out to be the most valid and sensitive test in the evaluation of iron deficiency in patients with active RA. If s-ferritin values of 14-50 μg/l are found iron deficiency may be overlooked if one uses the usual lower limit of 14 μg/l. Other authors used a c.o.p. of 60 μg/l [1,2] and 45 μg/l [8].

The combination of s-ferritin and MCV resulted in a specificity and predictive value of 100% (c.o.p. 50 μg/l and 80 fl, respectively)

which implies that iron deficiency is always present when finding this combination, but may be a little underdiagnosed (sensitivity 79%) if no bone marrow aspiration is performed. The addition of transferrin (c.o.p 50 μmol/l) to s-ferritin and MCV means proof for iron deficiency (validity of this combination was 100%).

The combination of blood parameters is useful in the assessment of iron deficiency, but a number of patients cannot be evaluated in this way because they have other combinations (e.g., s-ferritin less than 50 μg/l and a MCV more than 80 fl; see Table 3).

The erythrocyte-ferritin was of no value in the determination of iron deficiency in RA, confirming earlier results [10], though it is reported to be diagnostic in iron deficient patients without chronic disease [5].

The cause of the high prevalence of iron deficiency in RA found in our and other studies remains obscure. Since gastrointestinal blood loss, for instance due to the use of antirheumatic drugs, can occur subclinically and intermittently [13] this is not fully excluded in our patients. We found that iron absorption was highest among iron deficient patients. The negative correlation of iron absorption with RA activity found in ACD here, in which iron stores are present, might indicate that defective iron absorption is the result of active RA rather than a cause of ACD or iron deficiency.

Patients with a hypochromic microcytic anemia had a higher iron absorption, although not significant. This is explained by the fact that these changes in cellular indices are associated with iron deficiency. The validity of the iron absorption test for detecting iron deficiency was low.

Independent of its pathogenesis iron deficiency is considered to be protective in inflammation [14]. It is therefore questionable, after excluding all causes of iron deficiency, whether iron suppletion is indicated because it may exacerbate disease activity [15] though Hansen did not find deleterious effects of oral iron treatment in RA patients [1].

In summary, the present study showed a high prevalence of iron deficiency among anemic RA patients. ACD with and without iron deficiency was associated with a clearly different pattern of

erythrocyte and serological parameters. When iron deficiency is present in RA patients it generally coexists with ACD. Therefore the usual blood parameters should be adapted for ACD tend to direct MCV, transferrin and especially s-ferritin in an opposite way than iron deficiency does, while MCH is frequently lower than normal in ACD. The most specific and predicting parameter for the detection of iron deficiency was MCV, while s-ferritin is the most sensitive and valid test. Combination of MCV and s-ferritin raised specificity and predictive value to 100% and adding transferrin also raised sensitivity to 100%, though a number of patients are unevaluable using combinations. Iron absorption was shown to be higher in iron deficient patients. The iron absorption test and erythrocyte ferritin are of no value in determining iron deficiency in RA patients. Iron deficiency among RA patients with ACD can be detected accurately without bone marrow aspiration when normal ranges for blood parameters are adapted.

CHAPTER 5

PREDICTION AND EVALUATION OF THE EFFECT OF IRON TREATMENT IN ANEMIC RA PATIENTS

5.1 INTRODUCTION

In the studies described in Chapter 3 and 4 it was shown that iron deficiency occurred in 52-55% in anemic RA patients. It was demonstrated that iron deficiency was mostly associated with hypochromic microcytic anemia whereas ACD was associated with either normochromic normocytic or hypochromic normocytic anemia (Chapter 4). Overlap occurred, however, between iron deficiency and ACD in RA. We found that a MCV lower than 80 fl, a transferrin higher than 50 μmol/l and a serum ferritin lower than 50 μg/l predicted iron deficiency correctly. The ultimate proof of iron deficiency, however, rests upon a Hb rise following iron suppletion. Iron treatment of ACD in RA resulted in a transient Hb rise but in that study high parenteral doses were used [1,2]. Others saw no effect of iron treatment in RA patients with ACD [3].

Iron deficiency is considered to be protective in inflammatory diseases [4] and infection [5] while exacerbations of RA disease activity and synovitis are reported after iron treatment [1,2].

This study was performed to predict iron responsiveness of anemia in RA by means of simple erythrocyte and serological parameters and to evaluate whether iron treatment results in an increase of RA disease activity serologically.

5.2 MATERIALS AND METHODS

5.2.1 Demographic features

Twenty-eight patients (5 male) with definite or classical rheumatoid arthritis and a serum hemoglobin (Hb) of less than 7.4 mmol/l who did not receive iron treatment for at least six weeks previously, entered the study after giving written informed consent.

Other causes of anemia were excluded by history (present or past ulcer disease, other gastrointestinal disease or complaints, menorrhagia and hematuria), negative stools for occult blood, a negative Coombs test, a normal vitamin B12, folic acid, creatinin clearance and absence of microscopic hematuria.

Patients were classified according to stainable bone marrow iron content. Group I (no stainable iron), group II (0-1; normal to very small amount, group III [1-2] and group IV (3 or more). Groups I and II were considered iron deficient (6,7). The other classification was based on cellular indices (range for MCV 80-90 fl and for MCH 1600-2200 amol). Patients in group A were hypochromic microcytic, in B hypochromic normocytic, in C normochromic normocytic, in D normochromic microcytic and in E hyperchromic macrocytic. Mean age was 64 (48-79) years. A median disease duration of 4.2 (1-18) years was found, while 71% were seropositive (Rose test) with a mean titer of 261 (reciprocally). 61% Percent of the patients received long-acting antirheumatic drugs and 72% used NSAID's. These data, including age and sex did not differ significantly in the various subgroups.

5.2.2 *Laboratory procedures*

The following parameters, described in Chapter 2, were assessed:

Erythrocyte parameters:
Hb, MCV, MCH, reticulocyte count,
Iron status:
Serum iron, transferrin, ferritin,
Disease activity:
ESR, CRP and C1qba.
Iron absorption test.
Bone marrow aspirate (after sternal puncture) was stained for iron.

5.2.3 *Treatment schedule*

Patients were treated for 6 weeks with an daily dose of (oral) Fero-Gradumet (Abbott BV, Amstelveen, The Netherlands), containing 105 mg elementary iron. After 2 weeks reticulocyte count was repeated and

after 6 weeks all laboratory procedures were repeated except reticulocyte count, iron absorption and bone marrow aspiration.

5.2.4 Statistics

Sensitivity, specificity, validity and predictive value were calculated as described in 2.11.5.

5.3 RESULTS

5.3.1 Baseline characteristics in relation to bone marrow iron content

Sixteen patients (57%) were iron deficient (groups I and II). Table 1 and 2 show baseline characteristics and classification of the anemia in relation to cellular indices. Data and classifications at baseline were approximately the same as described in Chapter 4 (4.3.1 and 4.3.2).

5.3.2 Responsiveness of the anemia to iron treatment

A Hb increase after 6 weeks of treatment with ferrosulphate was found in 17 patients (61%). Median Hb increase was 0.6 mmol/l (range 0.1-1.8). In all 44 patients a median Hb increase of 0.3 mmol/l (range -1.2-1.8) was seen.

a) Relationship between bone marrow iron content and response to treatment

As is shown in Table 1 and 3 only in iron deficient patients (group I and II) a Hb rise occurred. The rise among responders was highest in group I but the response rate was lower than in group II.

After treatment 11 of the iron deficient patients (69%) were still anemic.

A slight Hb decrease was found among patients with ACD (III and IV). All these patients remained anemic after treatment.

TABLE 1 Relationship between bone marrow iron content (I=0, II=0-1, III=1-2, IV≥3), baseline (B) and data after (A) 6 weeks of iron treatment (values expressed as median and range). Age and sex did not differ in groups I-IV.

	I n=8 (28.6%)		II n=8 (28.6%)		III n=9 (32.1%)		IV n=3 (10.7%)	
	B	A	B	A	B	A	B	A
a) Erythrocyte parameters								
Hb (mmol/l)[1]	5.9 (4.2-6.5)	6.6* (6.0-7.9)	6.2 (5.4-6.7)	6.9^{+} (6.0-7.8)	6.3 (5.4-7.1)	6.2 (5.5-7.3)	6.7 (6.5-7.0)	6.2 (5.8-6.4)
MCV (fl)	80 (60-100)	85* (72-107)	81 (72-107)	87 (72-105)	86 (81-98)	82 (72-96)	84 (80-90)	83 (81-85)
MCH (amol)	1583 (1310-1730)	1566 (1370-1910)	1549 (1340-2080)	1623 (1280-2040)	1683 (1510-1950)	1665 (1520-1910)	1643 (1560-1780)	1547 (1520-1570)
Reticulocytes (⁰/00)[2]	21 (10-44)	31 (25-46)	24 (8-49)	32* (15-71)	25 (13-31)	36* (4-49)	26 (19-31)	36 (32-48)
b) Iron status								
Iron (μmol/l)	5 (2-7)	10* (3-21)	4 (1-8)	9** (3-16)	5 (3-10)	6^{+} (3-20)	5 (5-8)	5 (3-8)
Iron absorption	8.1 (0.8-10.5)		1.7 (1.0-11.0)		2.0 (0.8-3.5)		1.2 (0.8-1.4)	
Transferrin (μmol/l)	63 (40-81)	59 (36-75)	52 (33-62)	59 (33-73)	52 (45-60)	54 (44-63)	57 (52-65)	51 (44-62)
Ferritin (μg/l)	18 (10-58)	16 (10-63)	26 (10-119)	31 (10-55)	109 (21-247)	65* (17-153)	77 (45-126)	191 (18-363)
c) Disease activity								
ESR (mm/h)	75 (17-96)	48 (15-140)	82 (53-120)	54 (24-100)	84 (45-124)	70 (7-120)	49 (40-47)	68 (26-108)
CRP (mg/l)	21 (3-93)	27 (4-67)	34 (2-78)	10 (4-25)	30 (6-102)	43 (2-67)	23 (18-76)	37 (5-38)
Clqba (%)	14 (4-33)	13 (3-36)	27 (8-69)	34 (13-55)	25 (7-57)	29 (5-63)	26 (19-80)	15 (-)

1) Hb in mmol/l x 1.61 = g/dl.
2) Reticulocyte count was assessed before and after 2 weeks of iron treatment.
Significance level before vs after treatment: *p<0.10; **p<0.05; $^{+}$ p<0.01.

TABLE 2 Relationship between cellular indices (range for MCV 80-96 fl and for MCH 1600-2200 amol) and response to treatment. Values expressed as median with range.

	Hb (mmol/l)	Number of responders (%)	Hb rise[1] in responders (%)	Hb rise[1] in all patients(%)	Significance level[2]
Hypochromic Microcytic n=8 (29%) (A)	6.1 (5.4 - 6.7)	7 (88)	0.7 (12) (0.3 - 1.1)	0.6 (10) (0.0 - 1.1)	p<0.01
Hypochromic Normocytic n=8 (29%) (B)	6.2 (5.4 - 6.7)	4 (50)	0.3 (5) (0.1 - 1.0)	0.0 (0) (-0.2 - 1.0)	ns
Normochromic Normocytic n=10 (36%) (C)	6.6 (5.8 - 7.1)	4 (40)	0.2 (3) (0.1 - 1.2)	-0.1 (2) (-1.2 - 1.2)	ns
Normochromic Microcytic n=1 (3%) (D)	4.2	1 (100)	1.8 (43)	1.8 (43)	ns
Hyperchromic Macrocytic n=1 (3%) (E)	6.1	1 (100)	0.2 (3)	0.2 (3)	ns

1) Hb rise is written as absolute value and percentage of initial value in parenthesis.
2) P value was calculated from Hb change in all patients within a subgroup.

TABLE 3 Relationship between bone marrow iron content and response to treatment (I=0, II=0-1, III=1-2, IV=3). Values expressed as median with range.

	Responders	Hb rise[1] in responders	Hb rise[1] in all patients	Significance[2] level
I n=8	5 (63)	0.9 (15%) 0.3-1.8	0.5 (9%) (-0.3-1.8)	0.10
II n=8	8 (100)	0.6 (10%) (0.1-1.1)	0.6 (10%) (0.1-1.1)	0.01
III n=9	4 (44)	0.2 (2%) (0.1-0.2)	-0.1 (2%) (-0.9-0.2)	ns
IV n=3	0	0	-0.5 (8%) (-0.2-1.2)	ns

1) Hb rise in mmol/l written as absolute value and percentage of initial value in parenthesis.
2) P value was calculated from Hb change in all patients within a subgroup.

b) Relationship between cellular indices and response to treatment

Table 2 shows that a significant Hb rise is only found in patients with a hypochromic microcytic anemia, the response rate being 88%. Hb remained unchanged or decreased slightly in groups B and C. In D and E both patients responded.

5.3.3 Prediction of responsiveness of the anemia to iron treatment

MCV showed to have the highest specificity and predictive value for the prediction of a Hb rise (Table 4). At a cut off point of 80 fl sensitivity was low, however, it correlated strongly with a Hb rise.

MCH had a little higher sensitivity but validity did not differ from MCV.

Combination of MCV and MCH resulted in a higher validity compared to MCV and MCH separately (71 vs 61%).

The iron absorption test was not predictive for a Hb rise (not shown).

Transferrin was a sensitive but not specific test in the prediction of a Hb rise.

Combination of transferrin and MCV resulted in a higher validity (83 vs 61%).

Ferritin was the most valid single blood parameter. It was found to correlate negatively with a Hb rise.

Combination of ferritin and MCV resulted in a 100% specificity and predictive value but sensitivity was lower than ferritin alone.

5.3.4 Effect of iron treatment on erythrocyte and serological parameters

a) Change in erythrocyte parameters

MCV rise was associated with Hb rise ($r=0.71$; $p<0.0025$) and s-iron ($r=0.61$; $p<0.0025$). Hb change did not correlate with changes in other parameters. Table 1 shows that MCV tended to increase in group I and II after treatment.

TABLE 4 Sensitivity, specificity, validity and predicitive value of erythrocyte and serological parameters for the prediction of a Hb rise.

	Sensitivity (%)	Specificity (%)	Validity (%)	Predictive value (%)	Coefficient of correlation[1)]
MCV < 80 fl	41	90	61	88	r=-0.52;p<0.005
MCH < 1600 amol	65	55	61	69	r=-0.25;ns
MCV < 80 fL MCH < 1600 amol	64	83	71	88	
Transferrin > 50 μmol/l	82	33	61	70	r=0.24;ns
Transferrin > 50 μmol/l MCV < 80 fL	88	75	83	88	
Ferritin < 50 μg/l	73	82	78	85	r=-0.49;p<0.01
Ferritin < 50 μg/l MCV < 80 fL	64	100	88	100	

1) Correlation of initial value of parameter with Hb rise.

TABLE 5 Change in cellular indices after iron treatment in relation to Hb response rate. Initial classification is shown on the left vertical axis. Classification after treatment on the horizontal axis. (Normal range for MCV 80-96 fl and for MCH 1600-2200 amol).

	Hypochromic microcytic number(%)	Hb rise number(%)	Hypochromic normocytic number(%)	Hb rise number(%)	Normochromic normocytic number(%)	Hb rise number(%)	Normochromic microcytic number(%)	Hb rise number(%)
Initial classi-fication								
Hypochromic Microcytic n=6[1)] (A)	5 (83)	5 (100)	1 (17)	1 (100)	0		0	
Hypochromic Normocytic n=7[2)] (B)	1 (14)	1 (100)	3 (43)	0	3 (43)	2 (67)	0	
Normochromic Normocytic n=10 (C)	0		4 (40)	0	4 (40)	3 (75)	2 (20)	1 (50)

1) In 2 patients indices were not reassesed.
2) In 1 patient indices were not reassessed.

MCH increased in II but decreased significantly in I and II after treatment.

In group A cellular indices remained unchanged in the majority (Table 5). All patients, including the one who became normocytic, had a Hb rise.

In group B a slight Hb rise was only found in 2 of 3 patients whose MCH turned to normal and in 1 in which MCV decreased.

In group C a small Hb increase was observed in 3 of 4 patients when indices did not change and in 1 of the 2 in which MCV decreased.

In group D and E indices were unchanged after treatment with a Hb rise in both patients.

The reticulocyte count 2 weeks after initation of treatment tended to rise in all groups (Table 1).

b) Change in parameters of iron status

S-iron rose in group I, II and III. No correlation was found between s-iron rise and iron absorption. Transferrin did not change significantly after treatment. Ferritin decreased in group III and II while an unsignificant increase was seen in I and IV (Table 1).

Ferritin change was inversely correlated with transferrin change ($r=0.56$; $p<0.0025$) but not with Hb change.

c) Change in parameters of disease activity

ESR decrease in group I and II separately was not significant (Table 1). If they were taken together (iron deficient patients) the decrease was significant ($p<0.05$). In III and IV no significant change was seen.

CRP and Clqba did not change significantly in the various subgroups.

Ferritin change correlated with ESR change in groups III and IV together ($r=0.83$; $p<0.05$). It correlated with CRP change in I and II together ($r=0.88$; $p<0.0005$) and in III and IV together ($r=0.70$; $p<0.025$). In the case of Clqba a correlation was only seen in groups I and II ($r=0.60$; $p<0.025$).

Hb change correlated with a change in ESR in all patients ($r=0.34$;

$p<0.05$) but not in groups I and II alone. No correlation existed between CRP and C1qba change with Hb change.

In Table 6 it is shown that in iron deficient patients no differences in Hb increase were seen between patients in which parameters of disease activity either remained unchanged (decrease less than 10%) or increased on one hand and patients with a decrease in these parameters on the other. The same was found for a Hb decrease in groups III and IV.

TABLE 6 Relationship between Hb change and change in ESR, CRP and C1qba (Hb values expressed as median with range).

	I and II Hb change (mmol/l) (number)	III and IV Hb change (mmol/l) (number)
ESR =/↑	0.6 (0.1-1.8) (n=8)	0.5 (-1.2-0.1) (n=5)
ESR ↓	0.5 (0.2-0.9) (n=8)	0.0 (-0.6-0.2) (n=7)
CRP =/↑	0.8 (0.3-1.8) (n=9)	-0.2 (-1.2-0.2) (n=8)
CRP ↓	0.5 (-0.1-1.0) (n-7)	-0.3 (-0.9-0.2) (n=4)
C1qba =/↑	0.7 (0.2-1.4) (n=11)	-0.2 (-0.4-0.2) (n=10)
C1qba ↓	0.6 (0.2-1.8) (n=5)	-0.2 (-1.2-0.2) (n=2)

=/↑ means less than 10% decrease or increase
↓ means more than 10% decrease

5.4 DISCUSSION AND SUMMARY

This study was performed to predict iron responsiveness of anemia in RA by means of simple erythrocyte and serological parameters and to evaluate whether iron treatment results in increase of serological parameters of disease activity.

We found that 57% of patients were iron deficient which is in agreement with a prevalence earlier described (Chapter 3: 52% and Chapter 4: 55%).

Most iron deficient patients had a hypochromic microcytic or hypochromic normocytic anemia and those with ACD presumably had a normochromic normocytic or a hypochromic normocytic anemia as well as values from serum iron status and parameters of disease activity. These patterns did not differ from those described in the study in Chapter 4, which is to be expected since most patients studied here were derived from a subset of patients described in Chapter 3 and 4.

5.4.1 Responsiveness of the anemia to iron treatment

Only iron deficient patients had a significant Hb rise after 6 weeks of iron treatment. The response rate was 81% and in these patients median Hb increase was 0.8 mmol/l. The response rate was highest among patients with a hypochromic microcytic anemia (88%; Hb rise 0.7 mmol); 2 other iron deficient patients with different cellular indices also responded. Reassessment showed no other deficiencies, however, in these patients. Others found a response rate of 80% in iron deficient RA patients who were treated for 3 months [3]; the response rate therefore seems not to increase after prolonged iron therapy. Hb increase was higher in this study, however (1.5 compared to 0.8 mmol/l in our study). After treatment 69% of iron deficient patients still were anemic in our study. Continuation of treatment after 6 weeks possibly might have resulted in a further Hb increase. It must be realized, however, that most of all anemic and iron deficient patients probably also have features of ACD which may limit a maximal Hb increase. On the other hand, 83% of patients initially classified as having a hypochromic microcytic anemia had the

same indices after treatment, suggesting that they were still iron deficient. MCV and MCH did not change significantly after treatment. A problem in the evaluation of iron treatment is that the longer the patients are treated with iron the higher the chance that disease activity will decrease when patients have antirheumatic treatment simultaneously. So, if disease activity is decreased - which is associated with a Hb rise [8] - it is difficult to establish the role of iron treatment.

In patients without iron deficiency and most patients with normal cellular indices there was no significant Hb rise and all remained anemic. Most of patients with normal indices at baseline did not change their indices or became hypochromic. In groups III and IV a slight overall Hb decrease was found. In some patients Hb decreased more than 10%. In these patients ESR increased and MCH decreased. Since severity of ACD and hypochromasia are correlated with disease activity to some extent [8,9,10] this could explain the Hb decrease in these patients. A temporary rise in Hb in RA patients with ACD following iron treatment was found by others [1,2] but they used high parenteral doses of iron.

5.4.2 Change in parameters of disease activity and iron status

Parameters of disease activity tended to decrease in most patients after 6 weeks but this was only significant in groups I and II. In group III and IV a Hb decrease and ESR increase were strongly correlated, explaining most of the negative correlation between Hb and ESR change since this correlation was not found in iron deficient patients. CRP and C1qba changes were not correlated with a Hb change. Furthermore, it was shown that Hb changes were the same in patients with a decrease or increase in parameters of disease activity (Table 6). So, although a decrease in disease activity is associated with a Hb rise [8] it played a minor role in this study.

S-iron rose in all groups except IV but obviously this increase resulted in a higher bone marrow availability only in iron deficient patients, since no Hb rise was found in III and IV. Ferritin change, although not consistent, correlated with changes of ESR and CRP in ACD patients confirming its acute phase behavior [11]. Transferrin did not

decrease in iron deficient patients probably because these patients already had features of ACD masking a further decrease after iron treatment (Chapter 3,4).

In contrast to what others reported [2] we could not demonstrate any harmful effect of 6 weeks of iron treatment on serological parameters or disease activity in RA patients with or without iron deficiency.

The reticulocyte count after 2 weeks was higher in all groups suggesting an increased erythropoiesis. It was not correlated with a Hb rise after 6 weeks, however. Possibly during the initial phase of iron treatment a temporary increased bone marrow iron availability is present, even in ACD patients, while it is trapped by the mononuclear phagocyte system [12,13,14] in a later phase in patients with ACD.

5.4.3 Prediction of response to iron treatment

MCV turned out to be the most specific (90%) and predictive (88%) parameter of a Hb response. Using grade 0-1 stainable bone marrow iron as a standard Hansen [3] found a predictive value of 83%. Sensitivity was low. MCH alone was not useful while the combination of MCV and MCH resulted in a raised sensitivity (64%) and validity (71%) but both are still low in the prediction of a response to treatment. Sensitivity of transferrin (82%) was reasonable but predictive value (70%) and specificity (33%) were low. The combination of a specific test (MCV) with a sensitive test (transferrin) resulted in a reasonable validity (83%). S-ferritin was the most valid single test (78%). Its predictive value was 85% which is comparable to what Hansen [3] found (83%). The combination of MCV and s-ferritin proved to be useful. Both specificity and predictive value were 100%. This means that if MCV is less than 80 fl and s-ferritin less than 50 μg/l the patient will definitely respond to iron treatment. On the other hand if both values are above the cut off points some patients will also respond (sensitivity of 64%). It should be realized, however, that a combination of parameters restricts the number of patients to be evaluated with these parameters because different combinations occur. (eg. 1 parameter above and 1 parameter under the cut off point).

In summary, in this study it confirmed that many anemic RA patients have concomitant iron deficiency (diagnosed by means of staining a bone marrow aspirate for iron). Only patients with iron deficiency and cellular indices associated with iron deficiency show a significant Hb rise after 6 weeks of treatment with iron.

In the prediction of a Hb rise following oral iron administration MCV is the most specific and predictive single bloodparameter. Iron absorption was higher in iron deficient patients compared to patients without iron deficiency, but this test did not predict a Hb rise. Transferrin is the most sensitive test while ferritin is the most valid test. Combination of MCV and ferritin predicts a response in all patients although some patients with values above the cut off point of these parameters also respond. Bone marrow aspiration therefore rarely is indicated.

After 6 weeks the majority of patients are still anemic which suggest that either treatment was too short or the presence of ACD limited a further increase. Some parameters of disease activity tended to decrease after 6 weeks but after statistical analysis this turned out to be a minor contribution to the Hb rise. This also implies, however, that iron treatment did not result in increased serological RA activity.

CHAPTER 6

IMPAIRED IRON UPTAKE AND TRANSFERRIN BINDING BY ERYTHROBLASTS IN ANEMIA IN RHEUMATOID ARTHRITIS

6.1 INTRODUCTION

As was shown in Chapter 1 many factors are associated with ACD. Iron metabolism has been extensively studied in RA patients with ACD. It was first thought that decreased iron absorption might underly the development of ACD [1]. We found that iron absorption in nonanemics did not differ from those with ACD [2], whereas in iron deficient RA patients it was increased (Chapter 4, 5). In patients with ACD iron stores are increased by definition so the impaired iron absorption found in ACD is rather the result of RA disease activity than a cause of ACD or iron deficiency in RA.

Iron treatment in RA patients with ACD does not result in increased erythropoiesis (Chapter 5). Apparently, since in ACD bone marrow MPS-iron is increased, iron incorporation into erythroblasts in ACD is blocked. Some authors speculated that the MPS retains iron in ACD [3] although this turned out to be unspecific for ACD [4]. The same held through for ineffective iron turnover which was first found in RA and ACD [5] but later in iron deficient RA patients [6]. In another study ^{59}Fe-haem release from cultured erythroblasts was shown to be increased suggesting ineffective erythropoiesis [7].

It is not known, however, whether iron incorporation and transferrin binding by erythroblasts is impaired in ACD which as such might explain a decreased erythropoiesis in RA and ACD. The method for measuring these parameters were described by Katz et al. [8] and Van der Heul et al. [9] and was shown to be reliable.

We therefore examined iron incorporation and transferrin binding by erythroblasts in healthy controls and RA patients with and without anemia in order to determine a potential pathogenetic role of these factors in RA and ACD.

6.2 PATIENTS AND METHODS

6.2.1 Patients

Bone marrow from 5 healthy bone marrow donors and 18 patients with definite or classical RA were studied after receipt of the patient's written informed consent.

Group I (n=5) consisted of bone marrow transplantation donors (healthy controls), group II (n=5) consisted of nonanemic RA patients, group III (n=6) of RA patients with ACD and group IV (n=7) of iron deficient RA patients. Mean age was 58 years, mean disease duration had been 7 years (3-16), 13 (72%) of them used long-acting antirheumatic drugs and 15 (83%) used nonsteroidal anti-inflammatory drugs. These characteristics did not differ significantly among the 3 RA groups. We excluded patients who had treatment with iron, vitamin B12 or folic acid and those with B12 or folic acid deficiency, a present or past ulcer history, hematuria, menorrhagia, possible occult fecal blood test, hemolysis (Coombs, haptoglobin) and a decreased creatinin clearance.

6.2.2 Laboratory procedures

The following parameters described in Chapter 2 were assessed:

Erythrocyte parameters:
Hb, Ht, reticulocytes, MCV and MCH.
Iron status:
serum iron, transferrin, iron saturation, ferritin.
RA disease activity:
ESR,CRP.
Bone marrow aspirate (posterior iliac crest puncture)
- assessment of stainable bone marrow iron
- preparation of cell suspension (20 ml bone marrow) for separation of mononuclear cells (2.9.2).
- asessment of iron incorporation and transferrin binding to erythroblasts (Chapter 2.10).

Table 1 Parameters of erythropoiesis in group I-IV. Data are expressed as median with range.

	Controls I (n=5)	Nonan/RA II (n=5)	ACD/RA III (n=6)	Fe-def/RA IV (n=7)
Hb (mmol/l)	8.1 (7.7-8.2)	7.7 (7.4-8.7)	6.7 (6.6-6.9)	6.5 (4.3-7.1)
MCV (fl)	93 (86-95)	93 (86-116)	90 (81-98)	85 (61-95)
MCH (amol)	1940 (1770-1950)	1890 (1820-2370)	1900 (1540-2040)	1690 (1090-1920)
Retics (°/oo)	16 (3-21)	10 (1-13)	24 (5-30)	15 (5-26)
Erythroblasts* (°/o)	3 (2-18)	8 (1-26)	12 (4-29)	5 (1-27)

* Percentage of erythroblasts reflects nucleated erythroid cells in a cell culture smear without Epo stimulation before iron and transferrin addition.

Table 2 Parameters or iron status and disease activity in groups I-IV. Data are expressed as median with range.

	Controls I (n=5)	Nonan/RA II (n=5)	ACD/RA III (n=6)	Fe-def/RA IV (n=7)
Iron (μmol/l)	9 (5-12)	8 (1-9)	9 (4-11)	4 (2-10)
Transferrin (μmol/l)	50 (48-52)	56 (46-76)	50 (41-58)	56 (52-81)
Iron sat (°/o)	18 (10-25)	12 (2-23)	16 (8-27)	6 (4-15)
Ferritin (μg/l)	18 (12-28)	33 (10-326)	117 (38-432)	16 (10-53)
ESR (mm/h)	26 (16-46)	36 (21-65)	60 (52-101)	57 (32-104)
CRP (mg/l)	4 (2-6)	22 (2-41)	28 (10-121)	20 (2-78)

6.3 RESULTS

6.3.1 Parameters of erythropoiesis (Table 1)

MCV and MCH were significantly lower in group IV compared to I and II (p at least 0.05).

Reticulocyte count and percentage of nucleated erythroblast did not differ significantly between the four groups.

6.3.2 Parameters of iron status and disease activity (Table 2)

Serum iron and iron saturation was slightly lower in group IV compared to I and III ($p<0.10$).

Ferritin tended to be higher in group II compared to I ($p<0.10$). It was higher in III compared to I, II and IV (p at least 0.01), while in IV it was lower than in II and III (p at least 0.05).

ESR was higher in group III compared to I and II ($p<0.01$); in IV it was also higher than in I and II (p at least 0.05). It correlated negatively with Hb in II and III ($r=0.78$; $p<0.05$).

CRP was higher in group III compared to I ($p<0.01$), II and III ($p<0.05$). It correlated negatively with Hb in II and III ($r=-0.67$; $p<0.05$).

6.3.3 Iron incorporation into and transferrin binding to erythroblasts (Table 3A and 3B; Figure 1A and 1B)

Iron incorporation was lower in group III compared to I and II at all times (p at least 0.05). It tended to be lower in III compared to IV at 30, 60 and 120 minutes ($p<0.10$). In IV it was only lower compared to I at 30 and 60 minutes ($p<0.05$) whereas it did not differ from II. The difference between I and II was not significant. A negative correlation was found between iron incorporation and CRP in group III ($r=-0.93$; $p<0.01$). Iron incorporation correlated positively with serum transferrin in groups II and III ($r=0.40$; $p<0.02$).

Transferrin binding was lower in group III compared to I at all times (p at least 0.05). The differences between III and II and IV

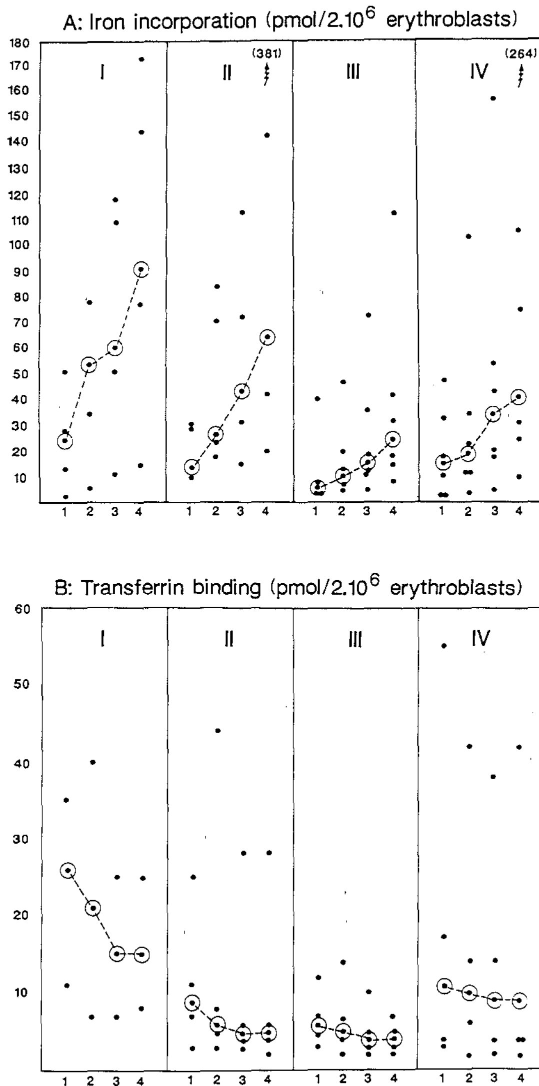
were not significant. In I it was higher compared to II at 2 minutes ($p<0.05$) whereas at 30, 60 and 120 minutes it tended to be higher ($p<0.10$). Transferrin binding correlated positively with serum transferrin in groups II and III ($r=0.55$; $p<0.05$).

Table 3 *Iron incorporation into (A) and transferrin binding to (B) erythroblasts in groups I-IV. Data are expressed as median range.*

	Controls I (n=5)	Nonan/RA II (n=5)	ACD/RA III (n=6)	Fe-def/RA IV (n=7)
A: Iron incorporation (pmol/2x10^6 cells)*				
1	25 (3-52)	14 (10-30)	6 (4-40)	16 (3-48)
2	55 (6-79	27 (18-85)	11 (5-47)	20 (4-102)
3	61 (11-119)	44 (15-114)	11 (5-73)	35 (5-158)
4	92 (15-174)	65 (20-381)	25 (9-113)	41 (10-264)
B: Transferrin binding (pmol/2x10^6 cells)*				
1	26 (11-35)	9 (3-44)	6 (3-12)	11 (3-55)
2	21 (7-40)	6 (3-44)	5 (2-14)	10 (2-42)
3	15 (7-25)	5 (3-28)	4 (2-10)	9 (2-38)
4	15 (8-25)	5 (2-28)	4 (2-10)	9 (2-42)

* Iron incorporation and transferrin binding per 2x10^6 erythroblasts. (1=2 min, 2=30 min, 3=60 min, 4=120 min after addition of ^{59}Fe-^{125}I transferrin).

FIGURE 1 Iron incorporation into nucleated erythroblasts (A) and transferrin binding by transferrin (B) (1= 2 min, 2= 30 min 3= 60 min and 4= 120 min after addition of ^{59}Fe-^{125}I transferrin). Median values are encircled and connected by broken lines.



6.4 DISCUSSION AND SUMMARY

Iron metabolism has been extensively studied in ACD and RA. Decreased iron absorption, once thought to be cause of ACD or iron deficiency [1], was later shown to be a secondary phenomenon of active RA [2,10]. Decreased iron availability, due to an impaired iron release by the MPS is another postulated theory [3] but in later studies it was found not to be related to ACD [4]. Various methods have been used to show that ineffective erythropoiesis, i.e. ineffective iron turnover by bone marrow, is a pathogenetical factor in ACD [5]. However, in other studies it was also associated with iron deficiency [6]. Williams et al. [7] found that ^{59}Fe-haem release, as a parameter of ineffective erythropoiesis, was increased in patients with RA and ACD and not in nonanemic or iron deficient RA patients. Their results, however, do not discriminate between impaired Hb synthesis or reduced erythroid precursor survival.

About iron uptake and transferrin binding by erythroblasts in RA and ACD no data exist. We therefore examined a potential pathogenetical role of decreased iron uptake and transferrin binding by erythroblasts in patients with RA and ACD. The method for measurement of these parameters, using ^{59}Fe bound to ^{125}I-transferrin, was shown to be reliable in previous studies [8,9,11,12].

We found iron incorporation by erythroblasts to be significantly reduced in patients with RA and ACD compared to healthy controls and nonanemic RA patients. It was shown that it correlated negatively with CRP, a RA disease parameter, in ACD patients which implies that the decrease in iron incorporation might be directed by RA disease activity. These findings suggest that decreased iron incorporation into erythroblasts might be a pathogenetical factor in ACD. Iron incorporation in ACD was also lower than in iron deficient RA patients but less clear. The difference might have been masked by the fact that disease activity was approximately similar in these two groups. From these data, however, it cannot be differentiated whether iron transport through the erythroblast membrane is impaired or whether iron is retained by monocytes or macrophages which are present in the medium. It is postulated that iron incorporation into erythroblasts

takes place through transferrin-receptor mediated endocytosis [13,14]. The iron is released from transferrin at the receptor site after which it is transported to the mitochondria [15]. Other experiments point to iron release from transferrin located intracellularly in coated vesicles [16]. After this transferrin is recycled into the medium [16]. We therefore examined whether transferrin binding by erythroblasts could be impaired in ACD, resulting in reduced iron uptake. We found that transferrin binding was impaired in ACD compared to healthy controls whereas it tended to be decreased compared to nonanemic and iron deficient RA patients. It also tended to be reduced in the other RA groups compared to controls. So it might be speculated that impaired iron uptake by erythroblasts found in ACD is caused by decreased transferrin binding to the erythroblasts because this was significantly reduced in ACD, whereas in the other 2 RA groups it was only slightly reduced. It can also be argued that intracellular iron release from transferrin is reduced resulting in decreased iron availability for haemsynthesis. Iron retention by monocytes/macrophages in the medium seems less probable because we do not expect these cells to influence transferrin binding by erythroblasts. It is not known whether transferrin receptor expression, its affinity at the erythroblast membrane surface or the number of transferrin receptors is reduced in ACD. Possibly in RA and/or ACD transferrin glycosylation is different [17]. This might result in a change of transferrin receptor affinity and iron binding [18,19]. In our experiments this was ruled out because the same transferrin was added to the medium in all groups.

In summary, we found that iron uptake by erythroblasts was reduced in RA patients with ACD, and to a lesser extent in iron deficiency. Iron uptake correlated negatively with RA disease activity. Transferrin binding to erythroblasts was also reduced in ACD. We therefore conclude that an impaired iron uptake by erythroblasts, possibly due to a reduced transferrin binding by erythroblasts, might be a pathogenetical factor in ACD in RA.

CHAPTER 7

PART ONE. DEFECTIVE ERYTHROPOIETIN RESPONSIVENESS TO THE ANEMIA IN RA PATIENTS WITH ACD AND IRON DEFICIENCY

7.1.1 INTRODUCTION

In Chapter 3 it was clearly shown that levels of parameters of RA disease activity like ESR, CRP and Clqba were higher in ACD patients compared to nonanemic RA patients. In Chapter 3 and 4 it was found that levels of these parameters were higher among ACD patients compared to iron deficient patients. The observation of a negative correlation between ESR, CRP and Hb found in Chapter 4 was also found by Birgegard et al. [1]. The mechanism through which an increased RA disease activity level determines the degree of anemia is unknown. Some authors found that interleukin-1 could be the factor involved [2], whereas others speculate on a role of tumor necrosis factor alpha [3].

A defective response of erythropoietin has also been claimed to play a role in ACD pathogenesis but the findings are controversial [4].

We examined a potential pathogenetic role of a decreased erythropoietin responsiveness to anemia in RA patients with ACD.

7.1.2 PATIENTS AND METHODS

7.1.2.1 Patients

Eighteen anemic RA patients (12 with ACD, 6 with iron deficiency, 2 with coexistent vitamin B12 deficiency and 2 with coexistent folic acid deficiency) were studied. The 12 patients with ACD were the same as those with ACD in the study described in Chapter 3. The 6 iron deficient patients, who served as controls, consisted of a randomly taken subset of the iron deficient patients from the study described in Chapter 3.

The same in- and exclusion criteria were used (see Chapter 3, page 50). Patient characteristics including age, sex, disease duration and antirheumatic treatment used showed approximately the same distribution as described in Chapter 3 (page 50).

7.1.2.2 Laboratory procedures

The following parameters described in Chapter 2 were assessed:

Erythrocyte parameters
Hb, Ht, reticulocyte count, MCV, MCH.
Iron status
Fe, iron, transferrin, ferritin.
Vitamins
Vitamin B12, folic acid.
Disease activity
ESR, CRP, Clqba, rose titer.
Serum erythropoietin
Bone marrow was aspirated after sternal puncture and stainable bone marrow iron was assessed.

7.1.3 RESULTS

TABLE 1 Relationship between type of anemia, Hb and serum erythropoietin (Epo). Data are expressed as median and range.

	Iron deficiency[1]	ACD[1]	Low B12[2]	Low folic acid[3]
Number	9	5	2	2
Hb (mmol/l)	5.9 (4.2–7.1)	6.3 (5.4–7.1)	6.6 (6.5–6.6)	6.4 (5.8–7.0)
Epo (U/l)	60 (10–420)	15 (10–28)	25 (20–30)	20 (10–80)

[1] Patients with low B12 or folic acid were excluded
[2] One patient with iron deficiency, one with ACD
[3] Both patients with iron deficiency

Correlation of parameters of erythropoiesis, iron status, disease activity with serum Epo

The erythrocyte parameters, iron status, disease activity, vitamin B12 and folic acid levels were comparable to the data shown in Chapter

3, Table 1.

Serum Epo was assessed in 18 anemic patients (12 of them were iron deficient and 6 had ACD). It is shown in Table 1 that Epo level was higher in iron deficient patients compared to patients with ACD ($p<0.02$). In iron deficient patients 1 patient (8%) had a serum Epo below normal. One patient in the ACD group (17%) had a serum Epo below normal. No difference was found in Epo level between B12 deficient and non B12 deficient patients. Epo level was lower in folic acid deficiency patients (ns).

Epo level correlated negatively with Hb level ($r=-0.6$, $p<0.005$) but did not correlate with ESR, CRP or C1qba. It was found to correlate negatively with ferritin for the whole group ($r=-0.45$, $p<0.05$) but not in ACD alone.

7.1.4 DISCUSSION AND SUMMARY

In this study we examined a potential pathogenetical role of a decreased Epo responsiveness in ACD in RA patients.

Epo in most patients were higher than in healthy controls [5]. Obviously there is some response to the anemia in both ACD and iron deficiency.

Epo level was significantly lower in patients with ACD compared to iron deficient patients while Hb did not differ significantly between these groups. These two types of anemia cannot be fully separated in RA patients as was described in Chapter 3 and 4 which might have decreased the difference in Epo level in these groups. Patients with low B12 or folic acid had lower Epo levels but they were equally distributed between iron deficient and ACD patients making interpretation of this finding difficult together with the low number of cases. A negative correlation was found between Hb and Epo, most pronounced in iron deficient patients. In both groups only 1 patient had a serum Epo below normal (8% among iron deficient and 17% in ACD patients). Epo correlated negatively with ferritin. These findings suggest that the response to anemia caused by iron deficiency in RA is higher than to ACD.

Baer [4] also found lower Epo levels in ACD in RA. Birgegard and

Erslev [1,6], however, found higher Epo levels in anemic RA patients than nonanemic controls irrespective of the cause of anemia. ACD in RA was associated with highest levels of ESR, CRP and C1qba (Chapter 3, Table 1). Epo was lowest in ACD although no correlation was found between ESR and Epo in contrast to Birgegard [1]. It might still be speculated that a factor, correlated with disease activity, has a restrictive effect upon Epo production. Since the number of patients investigated was small, further study is warranted in order to examine the relation of Epo and iron stores and to compare Epo response between anemic and nonanemic RA patients.

In summary, we found that serum Epo was increased in anemic RA patients compared to healthy controls and that serum Epo was higher in iron deficient RA patients compared to those with ACD, whereas serum Epo correlation negatively with serum ferritin suggesting that Epo response to anemia was higher in iron deficient patients. In both groups Epo response was insufficient to correct the anemia.

PART TWO. ERYTHROPOIETIN RESPONSIVENESS TO THE ANEMIA IN RA PATIENTS WITH ACD AND IRON DEFICIENCY COMPARED TO NONANEMIC RA PATIENTS

7.2.1 INTRODUCTION

It was shown in the study described in part one of Chapter 7 that serum Epo was increased in both RA patients with ACD and iron deficiency compared to serum Epo levels in 30 healthy individuals. This rise in serum Epo obviously was insufficient to prevent anemia. In that study sera from nonanemic RA patients were not examined because of a limited availability of the assay. In comparing serum Epo from nonanemic and anemic RA patients Erslev [1,2] and Birgegard [3] found higher serum Epo levels in ACD whereas Baer [4] found lower levels in ACD. The role of a decreased Epo responsiveness in the pathogenesis of ACD is therefore not yet fully established.

We found that serum Epo was higher among iron deficient RA patients compared to those with ACD (Chapter 7, Part one) while serum Epo level

correlated negatively with serum ferritin.

This study was performed to compare Epo responsiveness between nonanemic and anemic RA patients in order to determine its potential role in ACD. The second aim was to confirm our earlier observations of increased Epo responsiveness in iron deficient patients compared to those with ACD.

7.2.2 PATIENTS AND METHODS

7.2.2.1 Patients

Thirty patients (6 male, 24 female) with definite or classical RA were studied after giving written informed consent. Patients who were treated with iron, vitamin B12 and folic acid were excluded. Other causes of anemia were excluded by history, serum vitamin B12 and folic acid assay, the presence of hematuria or positive occult blood test in stool, decreased creatinine clearance and the presence of hemolysis, low B12 or folic acid. Patients were divided into three groups. Group I: 11 nonanemic patients; Group II: 7 iron deficient patients; and group III: 12 patients with ACD.

Overall disease duration was 8.5 years (5.2-14.6), 72% were Rose positive and the majority of patients (73%) were treated with long-acting antirheumatic drugs and/or nonsteroidal anti-inflammatory drugs. Mean age was 59 years (32-71). These clinical data did not differ significantly between the three groups.

7.2.2.2 Laboratory procedures

The following parameters, described in Chapter 2, were assessed:

Erythrocyte parameters
Hb, Ht, reticulocyte count, MCV, MCH.
Iron status
Fe, iron, transferrin, ferritin.
Disease activity
ESR, CRP, C1qba, rose titer.

Serum erythropoietin

Bone marrow aspirations (sternal puncture) were stained for iron content.

7.2.3 RESULTS

7.2.3.1 *Relationship between erythrocyte parameters, parameters of disease activity and serum Epo*

MCV and MCH were significantly lower in Group II and tended to be lower in Group III compared to Group I (Table 1).

ESR and CRP were significantly higher in Group III compared to Group I and II.

Epo in Group I and Group III did not differ and in Group II it tended to be higher compared to Groups I and II ($p<0.10$).

Hb correlated negatively with ESR ($r=-0.86$); $p<0.0005$) and CRP ($r=-0.39$; $p<0.05$). In Group II these correlations were not significant.

Epo correlated with neither erythrocyte parameters nor parameters of disease activity.

7.2.3.2 *Relationship between serological iron parameters and Epo*

Serum iron was significantly lower in Groups II and III compared to Group I (Table 2).

Transferrin was significantly lower in Group III compared to Group I.

Iron saturation was significantly lower in Groups II and III compared to Group I.

Serum ferritin was significantly lower in Group II compared to Groups I and III. It was highest in Group III.

Epo correlated negatively with serum ferritin in Group I ($r=-0.57$; $p<0.025$). No correlations were found between Epo and other parameters of iron status.

Table 1 Relationship between erythrocyte parameters, parameters of disease activity and serum Epo in group I (nonanemics), group II (iron deficiency) and group III (ACD). Data are expressed as median with range.

	I n=11	II n=7	III n=12
Hb (mmol/l)	8.0 (7.5–8.7)	6.6 (6.2–7.2)	6.4 (5.7–7.2)
MCV (fl)	91 (80–102)	87 (74–90)	87 (78–110)
MCH (amol)	1920 (1560–2070)	1770 (1400–1860)	1800 (1530–2390)
reticulocytes (0/00)	15 (5–39)	20 (6–30)	18 (5–41)
ESR (mm/h)	37 (21–60)	59 (14–105)	85 (34–102)
CRP (mg/l)	9 (4–45)	9 (2–57)	38 (10–74)
Epo (mU/ml)	41 (24–92)	50 (23–96)	43 (32–89)

Table 2 Serological parameters of iron status in group I (nonanemics), group II (iron deficiency) and group III (ACD). Data are expressed as median with range.

	I n=11	II n=7	III n=12
iron µmol/l	11 (5–17)	5 (3–7)	6 (9–11)
transferrin µmol/l	63 (47–78)	56 (51–64)	49 (39–67)
iron saturation %	16 (8–31)	9 (5–11)	10 (4–22)
ferritin (µg/l)	42 (18–142)	20 (10–24)	91 (36–273)

7.2.4 DISCUSSION AND SUMMARY

In this study we evaluated Epo response in anemic and nonanemic RA patients as well as the difference in Epo response between RA patients with iron deficiency and ACD.

Hb correlated negatively with parameters of disease activity (ESR and CRP) suggesting that, especially in ACD, RA activity dictates Hb level. This negative correlation was also found in previous chapters. Many theories exist to explain this correlation. Inhibition at stem cell level is claimed to play a role [5]. Interleukin-1 (IL-1) was able to suppress erythroid colony growth in vitro while serum IL-1 levels were higher in RA patients with ACD [6].

Serum Epo was determined using a sensitive radioimmunoassay [7]. The assay used in part one was different, so data of part one and two could not be combined. Furthermore in part one the availability of the assay was limited restricting the number of sera for Epo assessment.

In this study it was shown that serum Epo level was the same in nonanemics and ACD patients. It did not correlate with Hb level. Epo level was higher than in 30 healthy donors (22 $\pm$ 4 mU/ml). This suggests that in nonanemic RA patients an Epo rise effectively prevented development of anemia. Obviously when RA activity rises resulting in development of ACD, in which RA activity was found to be highest, Epo response is insufficient. In the previous study (Chapter 7, Part one) we did find a correlation with Hb and Epo in RA but more pronounced in iron deficiency than in ACD. We therefore concluded that some Epo response exists in RA and ACD but it is less prominent than in anemia of other etiology. Another possibility could be that bone marrow Epo sensitivity is impaired in RA and ACD but this cannot be concluded from these data. In vitro studies are necessary to demonstrate this phenomenon.

Epo was higher in iron deficient RA patients. The difference in Epo between these patients and patients without anemia or ACD is probably a little masked since these patients also have a certain level of disease activity resulting in features of ACD (Chapter 3 and 4). In the previous study (Chapter 7, Part one) Epo response in iron deficient RA patients was significantly higher compared to those with ACD. This was

supported here by the finding of a negative correlation between Epo and serum ferritin in nonanemics. These patients have a low degree of disease activity and therefore serum ferritin determines iron stores [8,9]. In Groups II and III serum ferritin levels reflect levels of disease activity more since it behaves like a positive acute phase reactant (Chapter 3, 4), so its relation with iron stores is less clear explaining the finding of only a weak negative correlation between Epo and serum ferritin in ACD. In our previous study (Chapter 7, Part one) the correlation between ferritin and Epo existed if all 18 subjects were considered together, but in ACD and iron deficiency separately the correlation was absent. In ACD associated with the highest degree of RA activity, serum Epo tended to be lower than in iron deficiency possibly due to mechanisms related to RA activity [3]. However, ESR was higher in iron deficiency compared to nonanemics while Epo was higher as well. This might point to a specific relation of Epo and iron status.

In summary, it was shown that in RA patients with or without anemia serum Epo is increased compared to healthy volunteers. In nonanemic RA patients this rise in Epo prevented development of anemia whereas in ACD - associated with a higher degree of disease activity - Epo response does not further increase and is therefore insufficient. In iron deficiency, with the same Hb level as in ACD, serum Epo tended to be higher. We conclude that a defective Epo response to anemia might play a pathogenetic role in ACD and Epo response might be negatively correlated with iron stores.

CHAPTER 8

THE EFFECT OF TREATMENT WITH THE ORAL IRON CHELATOR 1,2-DIMETHYL-3-HYDROXYPYRID-4-ONE ON ERYTHROPOIESIS AND SERUM EPO

8.1 INTRODUCTION

As was demonstrated in the study described in Chapter 6 iron uptake by erythroblasts and transferrin binding to erythroblasts is defective in ACD. Others examined ineffective iron turnover by erythroblasts in vitro and found it to be increased suggesting that iron release by erythroblasts is increased [1]. Other work points to a possible defective iron release by the MPS in case of ACD although this is controversial [2,3]. In both theories a decrease in iron availability for erythroblasts is supposed to be of pathogenetical importance in the development of ACD. Iron administration to RA patients with ACD for 6 weeks did not result in increased erythropoiesis suggesting that bone marrow iron availability does not increase in this way (Chapter 5).

Another observation was the possible defective Epo responsiveness to anemia in ACD whereas it was shown that in iron deficient RA patients Epo response was higher than ACD with the same degree of anemia suggesting a negative correlation of Epo response with iron stores (Chapter 7, Parts one and two). The use of desferrioxamine, a parenteral iron chelator was associated with a decrease in RA disease activity [4] and a Hb increase [5]. Recently an oral iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one (L1) was used successfully in thalassemia and transfusion hemosiderosis [6,7] and it was shown by Mostert et al. [8] not to be cytotoxic.

This study was performed to examine whether iron release from iron stores by oral iron chelation in RA patients with ACD results in increased erythropoiesis and Epo responsiveness to the anemia.

8.2 PATIENTS AND METHODS

8.2.1 Patients

Ten RA patients (2 male) with ACD fitting Cartwright's criteria [9,10] (2 male) were randomly selected from a group of 12 RA patients

with ACD from the study described in Chapter 7, Part two. Patients treated with iron, vitamin B12 and folic acid were excluded. Other cases of anemia were excluded by history, serum vitamin B12 and folic acid assay, the presence of hematuria or positive occult blood test in decreased creatinin clearance and the presence of hemolysis (Coombs, haptoglobin).

Overall disease duration was 9.2 years (5.2-18.3), 65% were Rose positive, whereas 79% used long-acting antirheumatic drugs and/or nonsteroidal anti-inflammatory drugs. Mean age was 62 years.

8.2.2 Treatment with L1

L1 was prepared as previously described [11] in gelatin capsules (0.5 g) and administered 1-2 hours before meals. Its structural formula is shown in Fig.1. The patients were treated for 1 week according to a protocol for use in RA patients (G.J. Kontoghiorghes; unpublished). At day 1, 2 g once a day, at day 2, 2 g in the morning and 1 g in the afternoon and from day 3 on 2 g twice daily were administered.

FIGURE 1 Structural formula of 1,2-dimethyl-3-hydroxypyrid-4-one (L1)

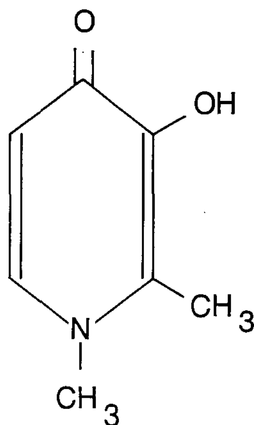


Table 1 Erythrocyte parameters, serum Epo and parameters of disease activity before and after 1 week LI treatment.

	Hb mmol/l	Ht	retics (0/00)	MCV (fl)	MCH (amol)	Epo mU/ml	ESR mm/h	CRP (mg/l)
before	6.0 (5.7–7.0)	0.30 (0.28–0.35)	18 (5–41)	87 (78–104)	1770 (1530–2150)	39 (21–67)	87 (22–102)	32 (4–70)
after	6.5^{+} (4.9–7.5)	0.32 (0.23–0.36)	22 (5–44)	88 (79–111)	1830 (1550–2310)	49^{+} (23–95)	84 (22–118)	17 (4–74)

$^{+}$ $p<0.10$. Data are expressed as median with range.

8.2.3 Laboratory procedures

The following parameters, described in Chapter 2, were assessed:

Erythrocyte parameters

Hb, Ht, MCV, MCH, reticulocytes.

Iron status

serum iron, transferrin, iron saturation (serum iron/transferrin), ferritin, urine iron excretion.

Disease activity

ESR, CRP.

Serum Epo

Bone marrow aspirations (sternal puncture) were stained for iron content.

All parameters, except bone marrow aspiration, were repeated after 1 week of treatment.

Table 2 Serological iron status before and after 1 week of L1 treatment (s-iron is serum iron, u-iron is urine iron excretion in 24 h).

	s-iron (μmol/l)	transferrin (μmol/l)	iron saturation %	ferritin (μg/l)	u-iron (μmol/24h)
before	6 (2–11)	51 (39–67)	11 (4–21)	91 (20–279)	0.5 (0.4–0.8)
after	14* (7–34)	61 (39–76)	24* (15–45)	50 (15–319)	4.4* (0.5–14.6)

*p<0.01. Data are expressed as median with range.

Table 3 Correlation between changes (Δ) in Hb, Epo, ESR, CRP and iron status after L1 treatment of ACD patients

Parameters	r	p
Δ Hb - Δ Epo	0.66	0.05
Δ Hb - Δ ESR	-0.76	0.01
Δ Hb - Δ CRP	-0.62	ns
Δ Hb - Δ iron saturation	0.53	0.05
Δ Hb - Δ ferritin	-0.78	0.01
Δ Epo - Δ ESR	-0.34	ns
Δ Epo - Δ CRP	-0.31	ns
Δ Epo - Δ ferritin	-0.76	0.025
Δ Epo - Δ iron	-0.48	ns
Δ Epo - Δ iron saturation	-0.31	ns
Δ Epo - Δ retics	0.48	ns

Ten paired data were analyzed. In case of CRP only 8 paired data were compared.

8.3 RESULTS

8.3.1 The effect of L1 iron chelation on erythropoiesis, disease activity and serum Epo

Hb increased in these 10 patients by 0.5 mmol/l ($p<0.10$). The response rate was 70%. Among responders the increase was 0.7 mmol/l ($p<0.02$) (Table 1).

Serum Epo increased nearly significant ($p<0.10$). A positive correlation was found between changes in Hb and Epo. Changes in ESR and Hb were correlated negatively (Table 3), although ESR did not change significantly.

8.3.2 The effect of L1 iron chelation on serum iron status and urine iron excretion

Serum iron, transferrin, iron saturation and urine iron excretion (in 24 h) increased significantly while a decrease in serum ferritin was found (Table 2).

Changes in Epo were negatively correlated with changes in serum ferritin (Table 3).

Hb increase was negatively correlated with serum ferritin change.

8.3.3 Tolerance to L1

No serious adverse reactions were seen in 10 patients treated with L1. Two patients had transient nausea (1 day). Endoscopic examination revealed no abnormalities. No changes were found in creatinin clearance, serum bilirubin, alkaline phosphatase, ASAT, ALAT and gamma-GT.

8.4 DISCUSSION

We examined whether iron release from iron stores in RA patients with ACD results in increased erythropoiesis and serum Epo. L1 was shown to be an effective oral iron chelating agent in RA patients with ACD. After 1 week serum iron, transferrin, iron saturation and urine iron excretion increased significantly while serum ferritin decreased. These findings confirm that in ACD in RA patients iron stores are increased [9,10 and Chapters 3, 4 and 5]. The drug was well tolerated with only little transient nausea occurring in 2 patients. In this limited number of patients and short period of treatment no obvious signs of toxicity occurred.

Hb increased in 70% of patients by 0.7 mmol/l after 1 week of treatment (0.5 mmol/l for the whole group). The increase correlated with serum ferritin decrease and increase of iron saturation. It is suggested that a decreased bone marrow iron availability due to a diminished iron release by the mononuclear phagocyte system (MPS) related to RA disease activity results in development of ACD [2,3]. We therefore believe that bone marrow iron availability is increased through a higher serum iron and iron saturation of transferrin after release of iron from the MPS and ferritin by L1 and its exchange with transferrin [12-14]. In the study described in Chapter 6 we found that iron uptake by and transferrin binding to erythroblasts was decreased in RA patients with ACD compared to those without anemia, iron deficiency and healthy controls. Since Hb increased this may very well

fit the concept that transferrin receptor expression on erythroblasts is increased by iron chelators resulting in more efficient iron uptake which was found by Louache et al. [15].

An increase in serum Epo was observed which was correlated positively with a Hb rise. If serum Epo change had not been related to treatment we would rather have expected a decrease in Epo as a Hb increase as such might diminish Epo demand. Hb and Epo changes correlated negatively with serum ferritin changes. Together with a higher Epo in iron deficient RA patients (Chapter 7, Parts one and two) these findings suggest that Epo response indeed is negatively correlated with iron stores. Others also found higher Epo levels in iron deficient RA patients [16] while Hb level did not differ from that in ACD. So the Hb increase following L1 treatment might not only be explained by factors described before but also by increased Epo response to the anemia. It is not known what causes the increased Epo response to the anemia after iron release. Rich et al [17] showed that Epo production occurs in bone marrow by macrophages and monocytes. The Epo production in bone marrow is dependent on local pH and oxygen tension [18,19]. Possibly Epo production or Epo gene expression by marrow macrophages increases when the increased iron stores in these cells are diminished but this is highly speculative. It was shown that in hemodialysis patients a Hb rise following desferrioxamine treatment occurred [19,20] but in this study it was not shown whether Epo response increased, although this is less probable because of insufficient renal production. In another study after prolonged desferrioxamine treatment in RA patients Hb decreased, but in this study baseline serum ferritin was low in most patients, suggesting iron deficiency, while Epo levels were not assessed [21].

Parameters of disease activity did not decrease significantly, although Hb rise correlated with ESR decrease suggesting that L1 treatment reduced RA activity resulting in a Hb rise. The period of treatment was too short for evaluation of effects of L1 on RA activity (both serologically and clinically), however.

Other studies point to a beneficial effect on RA activity using desferrioxamine as iron chelator [4,22]. Future studies with prolonged L1 treatment will possibly give further evidence for this effect.

In summary, we demonstrated that the administration of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) effectively chelated iron from iron stores. A Hb rise, seen in 70% of patients, correlated with a rise in serum Epo. After L1 treatment serum ferritin decrease correlated negatively with a Hb and Epo increase. These findings confirm our earlier observations of an increased Epo response in patients with lower iron stores. Iron release from iron stores might increase erythropoiesis in ACD through increased bone marrow iron availability, increased iron uptake and transferrin binding to erythroblasts or through increased Epo response to the anemia. Further study is warranted to establish the preliminary findings of increased erythropoiesis after iron chelation as well as safety of L1.

CHAPTER 9

THE ROLE OF TUMOR NECROSIS FACTOR ALPHA IN ANEMIA OF CHRONIC DISEASE IN RA PATIENTS: ELEVATED SERUM LEVELS AND IN VITRO SUPPRESSION OF ERYTHROPOIESIS

9.1 INTRODUCTION

In all studies described before we clearly found a negative correlation between parameters of RA disease activity like ESR, CRP and C1qba with Hb, especially among the RA patients with ACD. Levels of these parameters were higher in ACD compared to nonanemic and iron deficient patients. We found that iron incorporation into and transferrin binding by erythroblasts to be decreased in ACD while in iron deficient RA patients these were also slightly reduced (Chapter 6). It was also demonstrated that Epo response to anemia was insufficient, levels being the same in nonanemic RA patients and those with ACD while in RA patients with iron deficiency the response tended to be higher (Chapter 7, part one and two). Although these factors might contribute to the pathogenesis of ACD we searched for other possible factors which are related to RA disease activity and immune response and may act on erythropoiesis.

Interleukin-1, a cytokine produced by monocytes, was able to suppress in vitro erythropoiesis [1,2]. Tumor necrosis factor alpha (TNFα), another cytokine, which is frequently elevated in serum of patients with active disease [3,4] might also have a pathogenetic role in RA [5]. The effects of TNFα on erythropoiesis are uncertain [1,6,7]. We therefore studied the possible pathogenic role of TNFα in determining ACD in RA patients.

9.2 PATIENTS AND METHODS

9.2.1 Patients

Bone marrow from 33 patients with definite or classical RA [8] and 6 normal donors were studied after giving written informed consent: Group I (n=6) consisted of bone marrow transplantation donors (considered as

healthy controls). Group II (n=11) consisted of nonanemic RA patients, group III consisted of RA patients with ACD (n=13) (for definition see under "Laboratory procedures") and group IV consisted of RA patients with iron deficiency (n=9).

Patients who had iron, vitamin B12 or folic acid treatment recently or patients with a present or past ulcer history, hematuria, hypermenorrhoea, positive occult fecal blood test, hemolysis, vitamin B12 or folic acid deficiency or decreased creatinin clearance were excluded. From the RA patients 7 were male. Overall disease duration had been 8 years (range: 3-18), 71% used long-acting antirheumatic drugs (patients using corticosteroids or cytostatic drugs were also excluded) and 82% used nonsteroidal anti-inflammatory drugs. Mean age was 62 years. These characteristics did not differ in the three RA groups.

9.2.2 Laboratory procedures

The following parameters, described in Chapter 2, were assessed:

Erythrocyte parameters

Hb, Ht, reticulocyte count.

Iron status

Serum iron, transferrin and ferritin.

Disease activity

ESR, CRP, Clqba, Rose titer.

Serum TNFα

Bone marrow aspirate (posterior superior iliac crest puncture) was stained for iron content. Twenty milliliter of aspirate was used for culturing erythroblasts. BFUe was used as parameter for erythropoiesis (Chapter 2.9.2).

In group I (n=6), II (n=5), III (n=7) and IV (n=6) TNFα (Boehringer Institute, Vienna, Austria) 100 U/ml, TNFα 1000 U/ml and anti-TNFα (Boehringer Institute, Vienna, Austria) 1000 U/ml, respectively, were added to the BFUe cultures to evaluate their effects on in vitro erythropoiesis.

TABLE 1 Erythrocyte parameters, BFUe count, parameters of RA disease activity and TNF in healthy controls and RA patients without and with anemia (ACD or iron deficiency). Values expressed as median with range.

	I controls (n=6)	II nonanemic/RA (n=11)	III ACD/RA (n=13)	IV iron def./RA (n=9)
Hb (mmol/l)	8.1 (7.7–8.2)	8.0 (7.7–8.7)	6.4 (5.7–7.2)	6.5 (4.3–7.1)
Ht (L/L)	0.40 (0.37–0.40)	0.38 (0.35–0.42)	0.31 (0.28–0.36)	0.34 (0.24–0.37)
Reticulocytes (0/00)	16 (3–21)	10 (1–39)	17 (1–30)	12 (1–26)
BFUe count (per 10^5 cells)	346 (84–396)	305 (215–391)	189 (12–308)	179 (58–440)
ESR (MM/h)	21 (16–26)	36 (21–65)	76 (34–105)	62 (32–111)
CRP (mg/L)	4 (2–6)	22 (2–54)	63 (10–101)	22 (2–78)
C1qba (%)	3 (3–4)	5 (3–32)	14 (4–75)	13 (3–78)
Rose+	0	64 (0–512)	16 (0–256)	48 (0–512)
TNF (pg/ml)	7	13 (9–28)	28 (13–93)	14 (6–28)

+ Reciprocal Rose titre

TABLE 2 Serological parameters of iron status in healthy controls and RA patients without and with anemia (ACD or iron deficiency). Values expressed as median with range

	Controls (n=6)	nonanemic/RA (n=11)	ACD/RA (n=13)	iron def./RA (n=9)
Fe (μmol/l)	13 (12–14)	8 (1–15)	4 (2–11)	5 (2–10)
Transferrin (μmol/l)	52 (50–59)	56 (40–78)	46 (39–60)	60 (52–81)
Ferritin (μg/l)	22 (10–34)	33 (10–326)	125 (28–432)	13 (10–53)

9.3 RESULTS

9.3.1 Parameters of erythropoiesis, disease activity and serum TNFα in controls, nonanemic and anemic RA patients (Table 1)

Erythropoiesis. Hb and Ht did not differ between group III and IV (Table 1). Reticulocyte count did not differ significantly between the 4 groups. Hb correlated negatively with ESR and serum TNF (Fig. 1) and CRP ($r=-0.44; p<0.025$) and Clqba ($r=-0.46; p<0.01$) but not with Rose titer (groups I, II and III). BFUe counts were lower in group III compared to I ($p<0.05$) and II ($p<0.05$). They were also decreased in group IV compared to I ($p<0.05$). BFUe counts tended to be reduced in group II compared to I. The BFUe values correlated positively with Hb ($r=0.36$; $p<0,05$). Negative correlations were apparent between BFUe count and ESR (Fig. 1) but not with CRP, Clqba and serum TNF.

Disease activity. ESR, CRP and Clqba were higher in ACD compared to nonanemics (p at least < 0.05). ESR and CRP were higher in III compared to IV ($p<0.10$ and 0.05, respectively). Rose titer did not differ significantly between the 4 groups.

Serum TNFα was higher in group III compared to group II ($p<0.02$) and group IV ($p<0.10$). It correlated positively with ESR ($r=0.55$, $p<0.02$), CRP ($r=0.43$, $p<0.05$) and Clqba ($r=0.41$, $p<0.05$).

FIGURE 1 Correlation between ESR and Hb, ESR and BFUe count and serum TNFα and Hb level. Only data available from nonanemic RA patients and RA patients with ACD were used in order to avoid complicating factors involved (non-RA controls, iron deficiency).

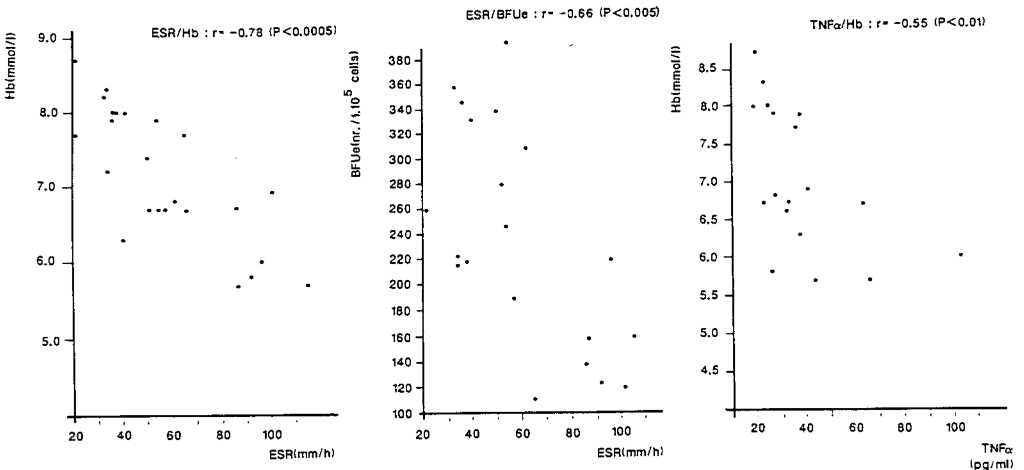


TABLE 3 Effects of the addition of TNFα and anti-TNFα antibody on erythroid growth from bone marrow in the four groups.

Addition to cultures	I controls n=6		II non-anemic/RA n=5		III ACD/RA n=7		IV Fe def./RA n=6	
	abs	%	abs	%	abs	%	abs	%
None	346 (92-396)	100	246 (239-349)	100	150 (12-280)	100	189 (58-440)	100
TNFα (100 U/ml)	287^{+} (66-348)	$82+7^{**}$ (71-89)	191^{**} (98-244)	$71+27^{**}$ (35-99)	86^{+} (2-195)	$57+20^{**}$ (17-78)	171 (98-441)	$81+12^{**}$ (65-100)
TNFα (1000 U/ml)	22^{**} (2-75)	$10+7^{**}$ (2-19)	0^{**} (0-29)	$4+5^{**}$ (0-12)	9^{**} (0-54)	$8+6^{**}$ (0-19)	8^{**} (5-22)	$2+2^{**}$ (1-6)
anti-TNFα (1000 U/ml)	394 (238-579)	$145+51^{+}$ (105-257)	418^{+} (218-469)	$136+29^{*}$ (90-180)	200^{+} (37-300)	$154+70^{+}$ (90-308)	208 (70-641)	$127+15^{**}$ (98-146)

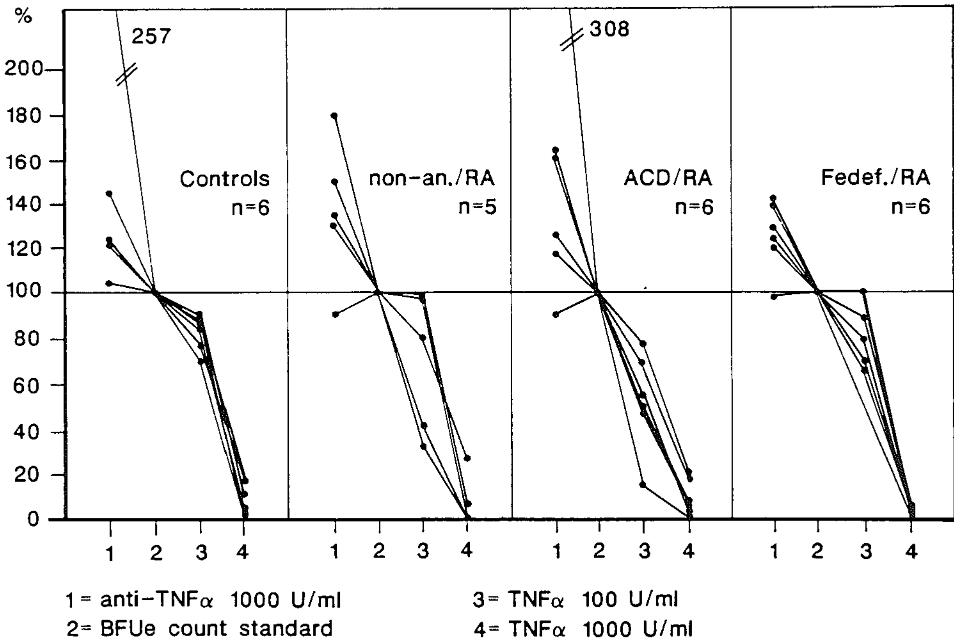
$^{+}$ $p<0.10$, * $p<0.05$, ** $p<0.01$ (compared to cultures with no addition)
Values indicate numbers of BFUe per 10^5 cells (median; range). Relative values (control set at 100%) are expressed as mean with standard deviation.

9.3.2 Correlation of iron status with parameters of erythropoiesis and disease activity and TNFα (Table 2)

Serum iron correlated positively with BFUe count (r=0.63, p<0.025) and negatively with serum TNF (r=-0.40, p<0.05), ESR (r=-0.38, p<0.05) and CRP (r=-0.36, p<0.10) in group III. Transferrin correlated positively with BFUe in groups II and III (r=0.38; p<0.05) and negatively with serum TNF (r=-0.53; p<0.01). Ferritin correlated positively with ESR (r=0.42, p<0.025), CRP (r=0.57, p<0.005) and serum TNF (r=0.38, p<0.05) but not with BFUe count.

9.3.3 Effects of addition of TNFα and anti-TNFα to bone marrow cultures (Table 3, Fig. 2)

FIGURE 2 The effect of addition of anti-TNFα and two concentrations of TNFα to BFUe cultures. Control-set is 100% in cultures with no addition (BFUe standard) in controls, nonanemic RA patients and RA patients with ACD or iron deficiency.



The addition of TNFα at a concentration of 100 U/ml significantly inhibited erythroid colony growth in group II ($p<0.02$) and slightly in III ($p<0.10$). Relative inhibition by 100 U/ml TNFα occurred in all groups, most pronounced in group III compared to group I ($p<0.05$) and group IV ($p<0.10$). A concentration of 1000 U/ml of TNFα inhibited growth in all groups.

The addition of anti-TNFα (100 U/ml) resulted in stimulation of erythroid growth in groups II and IV. In group I and III the same tendency was noted.

9.4 DISCUSSION AND SUMMARY

In this study we examined a potential pathogenetic role of TNFα in determining ACD in RA.

9.4.1 <u>Erythroid colony growth, parameters of disease activity and iron status</u>

An inverse relationship is apparent between Hb and parameters of RA activity, i.e. ESR, CRP and C1qba while these parameters were highest in ACD, confirming earlier findings in Chapter 3-5. Erythroid colony growth is impaired in RA patients with ACD and not in nonanemic RA patients. BFUe count correlated positively with Hb and negatively with ESR. Based on the experimental data we assume that inhibition of erythropoiesis of ACD in RA and the degree of inhibition is determined by RA disease activity. Others only found slight differences in BFUe count between RA patients with and without ACD [9,10], mainly because these studies lack sufficient numbers of bone marrows investigated.

ACD is associated with RA and several other chronic diseases. Harvey [9] found that BFUe count (from peripheral blood) correlated negatively with rheumatoid factor levels. We did not find this correlation confirming data from a previous study that rheumatoid factor plays no specific role in ACD pathogenesis (Chapter 3).

Iron metabolism has been studied extensively in ACD. It has been postulated that decreased iron availability to erythroblasts might be caused by decreased iron release by the mononuclear phagocyte system, but the idea is still controversial [11,12]. In Chapter 6 we found that

iron uptake and transferrin binding by erythroblasts was impaired in RA patients with ACD and to a lesser extent in iron deficiency. Because of this we included bone marrow of iron deficient anemic RA patients as an additional group since in the latter iron availability to marrow precursors is unequivocally decreased. BFUe growth was decreased to the same extent in iron deficient RA cases as in ACD. Although transferrin bound iron is added to the marrow cultures this amount obviously is not sufficient for erythroblasts from iron deficient patients. It seems, however, most likely that -in view of a higher RA disease activity level compared to nonanemics- also in these patients erythroid growth mainly is restricted by RA activity. In Chapter 6 we found that reduced iron uptake may be of pathogenetical value in the development of ACD. In this study the in vitro model does not provide information whether iron uptake by or iron availability to bone marrow cells is decreased in ACD. Based on data presented in Chapter 8 (increase in erythropoiesis after iron release) we believe this is the case and further evidence in support of a role of decreased iron availability comes from the fact that serum iron and transferrin correlated positively with BFUe numbers in RA patients with and without ACD. Transferrin is generally low and ferritin synthesis is augmented in active disease, resulting in iron trapping, hypoferremia and hence a lower iron transport to bone marrow [13,14]. Ferritin levels correlated positively with ESR, CRP and Clqba, confirming its acute phase behavior in active RA. No correlation of ferritin with Hb or BFUe marrow concentrates was found, however. Probably iron availability for marrow precursors does not further decrease after a certain level of ferritin synthesis, and hence iron entrapment, is reached. This might mask a potential negative correlation of ferritin with Hb and BFUe count.

9.4.2 TNFα and erythropoiesis

Another factor associated with disease activity, TNFα, formerly designated cachectin [3,4], is mainly produced by monocytes and macrophages [15] and may have a role in the pathogenesis of RA. One of the biological effects of TNFα is its ability to induce resorption of proteoglycan and inhibition proteoglycan analysis in cartilage [5] and it may therefore be of pathogenetical value in RA. TNFα levels have been shown to be elevated in synovial fluid from RA patients [16].

The results of this study show a positive correlation of serum TNFα with ESR, CRP and Clqba. This may suggest that TNFα may serve as a marker of disease activity in RA.

In the group with RA and ACD serum concentrations of TNFα were significantly higher than in the other groups. Hb correlated negatively with serum TNF. Of course these findings reflect increased disease activity of the ACD group. Elevated TNFα serum levels in ACD have -to our knowledge- not been described previously. A negative correlation between BFUe count and serum TNF did not appear, but it is not unlikely that this could be due to the wide variation in both parameters. In the iron deficient group serum TNFα level was similar as in nonanemics, suggesting that in these patients decreased iron stores contribute to the development of anemia.

In order to verify the potential pathogenetic role of TNFα in RA with ACD we added TNFα to the marrow cultures. Indeed TNFα exerted strongly and dose dependent suppressive effects on erythroid colony growth in controls, nonanemic RA patients, RA/ACD patients and iron deficient anemic RA patients. The relative inhibition by 100 U/ml TNFα was highest in the ACD group, suggesting increased susceptibility to TNFα. Lu [7] also demonstrated a suppressive effect on erythropoiesis by TNFα in serum free medium.

These observations suggest that a higher serum TNFα not only reflects disease activity but also that TNFα might play an important role in the pathogenesis of ACD in RA.

Several theories exist to explain the effects of TNFα on erythropoiesis. Possibly TNFα addition induces IL-1 production by monocytes [17], resulting in suppression of erythropoiesis [2]. Another possibility is a direct effect on CFUe and BFUe [6]. TNFα is able to decrease protein synthesis [18] and may theoretically inhibit Epo synthesis since Epo response to the anemia is insufficient in RA and ACD (Chapter 7). It might also be that TNFα reduces sensitivity of erythroblasts to Epo. Since IL-1 synthesis results in hypoferremia [19] while the secretion of IL-1 might be augmented by TNF addition [17]. Bone marrow iron availability could therefore be decreased by TNFα since we found that serum TNFα correlated negatively with serum iron and transferrin and positively with ferritin. Another speculation would be that TNFα might cause reduced iron uptake by and transferrin binding to erythroblasts which was found to be associated with ACD (Chapter 6).

To study whether endogenous TNF production by marrow cells contributes to the findings of decreased erythroid colony amounts in ACD anti-TNFα was added to the cultures. Indeed anti-TNFα upregulated colony growth but the effects were similar in all 4 patient groups. This confirms endogenous negative effects of TNFα produced by certain marrow cells in the medium but the phenomenon of TNF suppression did not differ among the 4 groups. It is suggested that a higher level of TNFα and subsequent marrow suppression in RA patients with ACD was not due to a higher production of TNFα by the marrow but in other sites, e.g., inflammatory sites such as the synovial membrane [16].

In summary, it was demonstrated that erythroid colony growth was reduced in RA patients with ACD. Since BFUe count correlated negatively with ESR it is suggested that RA disease activity affects erythroid colony growth in these RA patients. BFUe count was also reduced in iron deficient RA patients and since these patients had intermediate RA activity it was concluded that decreased iron stores was another important factor involved in these patients. Based on earlier studies (Chapter 6) we believe this or decreased iron uptake by erythroblasts may also play a role in ACD.

TNFα was found to correlate with parameters or disease activity and it correlated negatively with Hb but not with BFUe count possibly due to the wide variation in both parameters. It was significantly higher among RA patients with ACD. TNFα was able to suppress erythroid colony growth in vitro confirming a potential pathogenetic role in ACD. Anti-TNFα addition resulted in stimulation of erythroid colony growth suggesting that marrow cells in culture produce TNFα. The stimulatory effects of anti-TNFα were equal in all 4 categories suggesting that in vivo the effects of TNFα are attributable to TNFα production in other sites such as the joint and not to TNFα production in marrow cells.

CHAPTER 10

ANEMIA OF CHRONIC DISEASE IN RHEUMATOID ARTHRITIS. RAISED SERUM INTERLEUKIN-6 (IL-6) LEVELS AND EFFECTS OF IL-6 AND ANTI-IL-6 ON IN VITRO ERYTHROPOIESIS

10.1 INTRODUCTION

In Chapters 7-9 we studied the role of hematopoietic growth factors Epo and TNFα in the pathogenesis of ACD. Epo responsiveness to the anemia was shown to be reduced (Chapters 7 and 8). Bone marrow Epo sensitivity could also be impaired (Chapter 9). TNFα might have a pathogenetic role in RA [1]. We found that serum TNFα was elevated in RA patients with ACD while it inhibited erythropoiesis in vitro (Chapter 9).

Another interleukin, IL-6, is a monokine with biological activities related to inflammatory responses [2,3]. IL-6 levels were elevated in serum and synovial fluid of patients with active RA [4,5]. About the effects of IL-6 on bone marrow only few data exist [6].

We examined a possible role of IL-6 in the pathogenesis of ACD by comparing serum IL-6 levels and in vitro effects of IL-6 on erythroid progenitor cells in nonanemic RA patients, RA patients with ACD and healthy subjects.

10.2 PATIENTS AND METHODS

10.2.1 <u>Patients</u>

Bone marrow from 16 and serum from 21 patients (6 male, 15 female) with classical or definite RA and 5 normal donors were studied after patients' written informed consent. RA patients were divided into 2 groups: Group I consisted of 9 nonanemic patients and group II of 12 patients with ACD. Patients who had iron, vitamin B12 or folic acid treatment recently or patients with a present or past ulcer history, hematuria, menorrhagia, positive occult fecal blood test, hemolysis, iron, vitamin B12 or folic acid deficiency or decreased creatinin clearance were excluded. Patients using corticosteroids or

cytostatic drugs were also excluded. Overall disease duration had been 7 years [4-17], 74% used long acting antirheumatic drugs and 88% used nonsteroidal anti-inflammatory drugs. Mean age was 61 years. These characteristics did not differ between groups I and II.

10.2.2 Laboratory procedures

The following parameters, described in Chapter 2, were assessed.

Erythrocyte parameters
Hb, Ht, reticulocyte count.
Iron status
Ferritin.
Disease activity
ESR, CRP, Rose titer.
Serum IL-6
Bone marrow aspirate (posterior superior iliac crest puncture) was stained for iron content. Twenty milliliter of aspirate was used for culturing erythroblasts. BFUe was used as parameter for erythropoiesis (Chapter 2.8.2).

In 3 patients from group I, 8 from group II and in 5 normal donors IL-6 (1000 U/ml) and anti-IL-6 (1000 U/ml) (Central Laboratory for the Blood Transfusion Service, Amsterdam, The Netherlands) were added to the BFUe cultures to evaluate their effects on in vitro erythropoiesis. In 7 patients (group I: 2, group II: 5) twice the amount of IL-6 and anti-IL-6 was added.

10.3 RESULTS

10.3.1 Parameters of erythropoiesis, disease activity and IL-6 in RA patients with and without ACD

BFUe count was significantly lower in group II compared to group I (Table 1). Reticulocytes did not differ between the 2 groups.

TABLE 1 Erythrocyte parameters, parameters of disease activity, IL-6 and Burst Forming Units (BFUe) per 10^5 cells in RA patients without anemia and ACD.

	Hb mmol/l	Ht l/l	Retics 0/00	BFUe col. per 10^5 cells	ESR mm/hr	CRP mg/l	Rose reciprocal titer	IL-6	Ferritin (μg/l)
Nonanemic RA (n=9)	7.9 (7.5–8.3)	0.39 (0.35–0.42)	17 (5–39)	260 (215–391)	34 (21–60)	8 (4–45)	32 (0–256)	0 (0–11)	39 (12–86)
ACD RA (n=12)	6.6[1] (5.4–7.3)	0.31[1] (0.28–0.34)	17 (1–28)	191[2] (123–108)	74[1] (40–105)	62[2] (10–121)	32 (0–256)	29[3] (0–82)	108[2] (39–410)

Values are expressed as median with range. [1] $p<0.001$, [2] $p<0.01$, [3] $p<0.10$.

TABLE 2 Standard BFUe count (no addition; counts per 10^5 cells incubated) and absolute (abs) and relative (%) change in BFUe count after addition of IL-6 (1000 U/ml) and anti-IL-6 (1000 U/ml) to the cultures in controls, nonanemic and anemic RA patients.

Addition	Controls (n=5)		Non-anemic/RA (n=3)		ACD/RA (n=8)	
	abs.	%	abs.	%	abs.	%
None	356 (254–396)	100	246 (242–282)	100	173 (12–440)	100
IL-6	266[1] (225–266)	80+20[1] (57–105)	294 (168–318)	111+2[1] (109–113)	155 (6–471)	91+21 (83–107)
Anti-IL-6	219[2] (168–361)	72+16[3] (55–92)	228[1] (180–244)	86+10[1] (74–99)	135[1] (8–329)	72+13[3] (43–83)

Values expressed as median with range (percentages as mean and standard deviation).
[1] $p<0.10$, [2] $p<0.05$, [3] $p<0.01$; compared to standard BFUe count.

ESR and CRP were significantly higher in group II. Hb correlated negatively with ESR ($r=-0.71$; $p<0.001$) and CRP ($r=-0.68$; $p<0.005$). BFUe count correlated negatively with ESR ($r=-0.65$; $p<0.005$) and CRP ($r=-0.66$; $p<0.01$). No differences in Rose titer were found.

Serum IL-6 tended to be higher in group II. IL-6 correlated positively with ESR ($r=0.46$; $p<0.025$) and CRP ($r=0.52$; $p<0.025$) and negatively with Hb ($r=-0.41$; $p<0.05$) but not with BFUe.

10.3.2 *Effects of addition of IL-6 and anti-IL-6 to bone marrow cultures* (Table 2)

BFUe count was higher in controls compared to ACD patients ($p<0.05$). The difference between controls (healthy donors) and nonanemics was not significant.

IL-6 addition slightly inhibited BFUe count in controls but stimulated growth in nonanemics. In 7 cultures twice the amount of IL-6 was added (2 controls, 5 ACD; not shown). Mean percentual BFUe count was 109 ± 18% compared to control BFUe count.

Anti-IL-6 addition inhibited BFUe count in all groups, most pronounced in controls and ACD. Doubling the amount of anti-IL-6 added had no further inhibitory effect on BFUe count (mean 82 ± 16%; $p<0.01$; not shown).

10.4 DISCUSSION

In this study we examined a potential pathogenetic role of IL-6 in ACD.

Erythroid colony growth, measured by BFUe count, was impaired in patients with RA and ACD and not in nonanemic RA patients. Reticulocyte count was the same in nonanemic and anemic RA patients, which further points to an inadequate bone marrow response to the anemia. An inverse correlation was found between parameters of RA activity, i.e., ESR and CRP and BFUe count and Hb. These findings do not differ from those found in Chapter 9, which is not surprising since a randomly assigned subset of patients from Chapter 9 was examined here. In Chapter 9 we found that serum TNFα correlated

positively with ESR and CRP. It inhibited, whereas anti-TNFα stimulated, in vitro erythropoiesis, from which it was concluded that TNFα might be an important factor in the pathogenesis of ACD in active RA. In iron deficient RA patients decreased iron availability was another pathogenetical factor. In this study we excluded iron deficient RA patients.

IL-6 is a monokine with biological activities related to inflammatory responses [2,3]. It was shown that IL-6 levels were evaluated in serum and synovial fluid of patients with active RA [4,5].

Here we found that serum IL-6 levels correlated positively with parameters of RA activity, i.e. ESR and CRP, which confirms its role in the inflammatory response in RA. Serum IL-6 correlated negatively with Hb and it was elevated in most of our ACD patients. Since we found an inverse relationship between RA disease activity and BFUe count, it was assumed that IL-6, as TNFα (Chapter 9) might be a mediator of impaired erythroid colony growth.

IL-6 and anti-IL-6 antibodies were added to the bone marrow cultures in order to establish the effects on erythroid colony growth. IL-6 addition resulted in a slight inhibition of BFUe growth in controls and ACD. In nonanemic controls in contrast it stimulated erythroid growth. After addition of anti-IL-6 antibody erythroid colony growth was impaired in all cases. Addition of twice the amount of IL-6 or anti-IL-6 did not increase the in vitro effects of either two. From the data it is concluded that anti-IL-6 has inhibitory effects on erythroid colony growth. Probably IL-6 is produced by bone marrow monocytes in culture. Possibly the stimulatory effects of locally produced IL-6 might be neutralized by the addition of anti-IL-6 while IL-6 addition in the amount used here has no further effects on erythroid growth. The anti-IL-6 antibody specificity is not entirely known. So although a consistent inhibition of erythropoiesis occurred it is not known whether antibodies with a similar isotype might have caused the same suppression. It is for instance possible that it inhibits effects of other cytokines as well. IL-6 production is enhanced by IL-1 and TNFα [7,8,9]. It is therefore possible that IL-6 production in the marrow cultures is stimulated by IL-1 and TNFα production in the medium. These cytokines possess inhibitory effects

on erythropoiesis [10] (Chapter 9). In this way the potential stimulatory effects of IL-6 are masked in vitro. It might also be speculated that IL-6 triggers TNFα and IL-1 production resulting in nutralizing IL-6 effects as well. This might explain the inconsistent effects of IL-6 addition in the different groups. IL-6 has been found to trigger primitive hematopoietic cells into cell cycle in vitro, most pronounced in the presence of IL-3 [6], whereas others found that IL-6 had no effects on erythroid growth in acute myeloblastic leukemia [11]. Based on these preliminary data it seems unlikely that IL-6 -shown to be raised in ACD- is a cause of ACD in RA. In contrast, it might be speculated, based on the findings of anti-IL-6 addition, that IL-6 might counteract suppressive effects of other interleukins such as IL-1 [10] and TNFα (Chapter 9) on erythroid colony growth.

In summary, we found that serum IL-6 tended to be higher in ACD compared to nonanemics. It correlated positively with parameters of disease activity and negatively with Hb, suggesting a possible pathogenetical role in ACD. The effects of IL-6 addition to bone marrow cultures were inconsistent whereas anti-IL-6 addition resulted in decreased erythroid colony growth. From these preliminary data it was concluded that IL-6 plays no pathogenetical role in ACD; it might be speculated that it even counteracts suppressive effects of other interleukins like IL-1 and TNFα on erythroid growth.

CHAPTER 11

GENERAL DISCUSSION AND SUMMARY

In Chapter 1 the various causes of anemia in RA as well as the factors associated with ACD were discussed. It is clear that many causes must be excluded before the diagnosis ACD in RA can be made.In the discussion of factors like iron absorption, the role of the MPS, ineffective erythropoiesis and Epo it became clear that many of these studies lack proper description of the patients iron status. Factors like ineffective erythropoiesis and the iron retention by the MPS were first associated with ACD but later also with iron deficiency. In a number of studies on the role of Epo iron deficient patients were not sufficiently separated from ACD making interpretation of the results difficult. A number of mediators of erythropoiesis were discussed. Their effects on erythropoiesis are not consistent while only a few of them were specifically studied in ACD in RA. This thesis was performed to assess reliable methods to classify anemic RA patients which is inevitable for studies concerning pathogenesis of ACD. The other objective was to study the specific role in ACD of iron availability to erythroblasts and the effects of Epo, TNFα and IL-6 on (in vitro) erythropoiesis.

In Chapter 2 the various laboratory procedures as well as the statistical methods were described.

11.1 Diagnostic and therapeutic aspects of the anemia in RA (Chapter 3, 4 and 5)

In all studies presented here patients classification was based on stainable bone marrow iron content (1,2) in order to discriminate iron deficient patients from those having ACD. In combination with serum vitamin B12 and folic acid determinations it was shown that only 24% of RA patients have just one cause of anemia (ACD) while other subjects had combinations. If one relies on cellular indices only (MCV and MCH) many patients would have been classified as having ACD while in fact often a combination of iron and vitamin B12 or folic acid deficiency is present which has important diagnostic (workup for analysis of these deficiencies) and therapeutical consequences.

If all other causes of anemia are excluded the problem remains to detect iron deficiency if one wishes to avoid bone marrow examinations in all anemic RA patients. We found that MCV is the most specific and predictive parameter for the detection of iron deficiency. Ferritin is the most valid serum assay in this respect. Since ferritin is an acute phase reactant (3), clearly confirmed here because of a positive correlation with ESR and CRP, its lower reference limit must be elevated (from 14 to 50 μg/l) for the purpose of detection of iron deficiency. Ferritin level in iron deficiency is similar as in nonanemic patients for the same reasons since in iron deficiency RA activity was higher than in nonanemics. Serum transferrin (as TIBC) was even lower in iron deficiency than in nonanemics which is contradictory to what is found in uncomplicated iron deficiency (4) while ACD was found to be associated with a low serum transferrin and iron saturation. This shows that iron deficient patients also have features of ACD which is associated with the highest level of RA activity. This assumption was further confirmed by the finding that iron treatment of iron deficient RA patients results in only a limited Hb increase. RA activity, associated with ACD, probably determines maximal HB rise. In ACD patients no Hb rise occurred. The iron administered does not result in increased iron availability to erythroblasts and increased erythropoiesis possibly due to iron retention by the MPS or other pathogenetical factors. MCV and ferritin are reliable serum parameters to predict a Hb rise. The combination of the serum parameters MCV, transferrin and ferritin at their respective cut off points results in a 100% valid assessment of iron stores in RA patients while the combination of MCV and ferritin predicts iron responsiveness of the anemia in RA in 100% of cases.

It is concluded that anemia in RA has a multifactorial cause and complete evaluation is necessary for diagnostic and therapeutic consequences. Bone marrow aspiration is rarely necessary. In Fig.1a and 1b the approach of the anemic RA patients is shown schematically.

11.2 Pathogenetic aspects of ACD in RA

Impaired iron absorption -once thought to be of pathogenetical value in ACD [5]- was found to be lower in ACD using a simple iron

Figure 1A Causes of anemia in RA

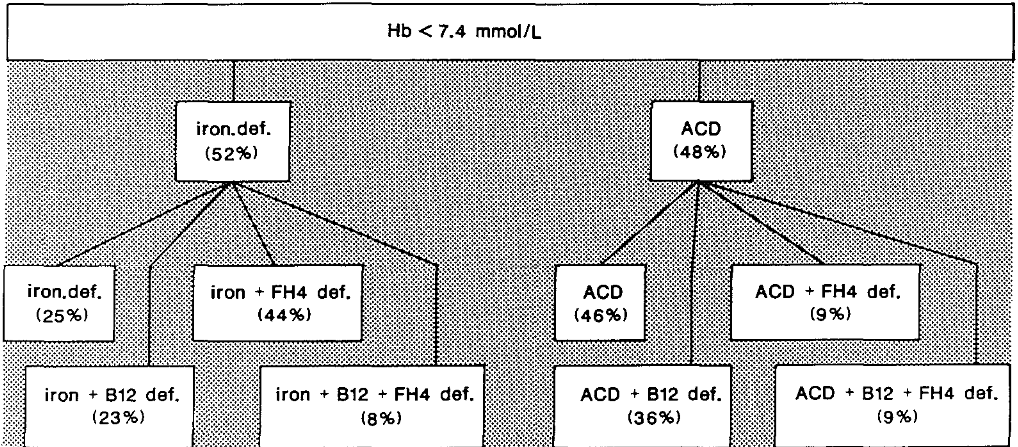
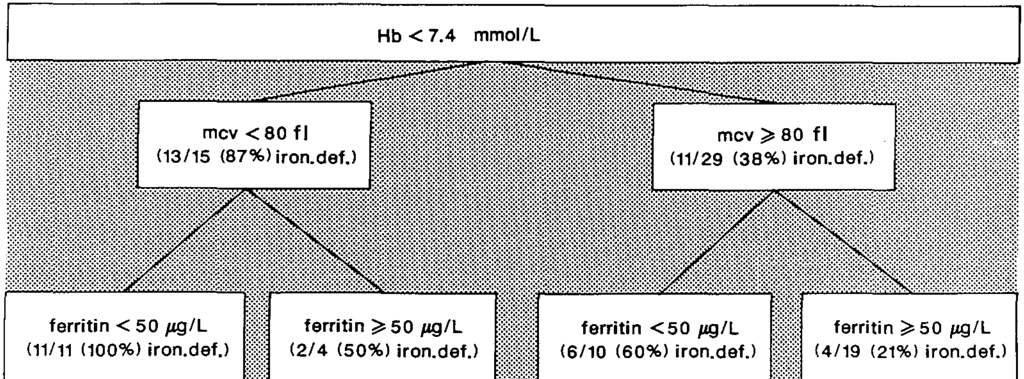


Figure 1B Predicition of iron deficiency in anemic RA patients using MCV and serum ferritin as key parameters



def. = deficiency, ACD = anemia of chronic disease, B12 = vitamin B12, FH4 = folic acid
(Percentages reflect the number of patients within the subgroup).

absorption test [6] (Chapter 4,5). In ACD iron stores are normal to elevated (measured as ferritin and stainable bone marrow iron) so a decreased iron absorption can not be the cause of ACD. Moreover, iron absorption was significantly higher in subjects without stainable iron. Therefore impaired iron absorption is rather the result of active RA and possibly a compensation to increased iron stores.

The increased iron stores found in ACD apparently are not available to bone marrow. Even treatment with iron does not result in increased erythropoiesis (Chapter 5). We therefore examined whether iron uptake or transferrin binding by erythroblasts could be impaired in ACD (Chapter 6). Indeed we observed that iron incorporation was reduced in ACD compared to nonanemics and healthy controls in ACD. Iron incorporation correlated negatively with CRP, an RA disease parameter, explaining the relatively small difference with iron deficient RA patients in which RA activity was approximately the same as in ACD in this subset of patients. The impaired iron uptake by erythroblasts might be caused by a significant decrease in transferrin binding to erythroblasts in ACD although considerable overlap occurred with the other RA groups. The findings confirm that iron availability to erythroblasts is reduced in ACD. It is not certain whether the phenomenon of reduced iron uptake and transferrin binding by erythroblasts is primary, i.e. causing the anemia, or secondary due to other factors involved in ACD pathogenesis like iron retention by the MPS [7] or hematopoietic growth factors discussed below.

We studied the role of Epo, and the cytokines TNFα and IL-6 in the pathogenesis of ACD in RA (Chapter 7,9,10). It was shown that serum Epo was increased in all RA patients compared to healthy controls using two different tests. Apparently in nonanemic RA patients this response is sufficient to prevent anemia. In part one of Chapter 7 a negative correlation between Hb and Epo was present most clearly in iron deficient patients. In part two of Chapter 7 this was not found. In ACD obviously no clear correlation exists between Hb and Epo confirming the insuffficent response to the anemia in ACD.

Another finding was a higher serum Epo in iron deficient RA patients compared to subjects with ACD while Epo level correlated negatively with ferritin in nonanemic patients. It was concluded that, beside RA disease activity (generally highest in ACD), increased iron

stores could inhibit Epo response to the anemia. A group of 10 RA patients with ACD therefore were treated with the oral iron chelator L1 to examine whether release of iron from iron stores results in increased Hb and serum Epo (Chapter 8). Iron was effectively chelated as shown by the rise in serum and urine iron, a rise in iron saturation of transferrin and a decrease of ferritin after 1 week of treatment. Both Hb and Epo tended to rise after 1 week treatment and a positive correlation was found between Hb and Epo change whereas changes in both parameters correlated negatively with ferritin changes. This suggests that Epo response to ACD may increase following iron release from iron stores and that it might cause a Hb rise although the findings are preliminary. It might also be that through increased iron exchange from transferrin to ferritin iron availability for erythroblasts increases, resulting in a Hb rise. Since it is postulated that transferrin expression increases following chelation therapy [8] it could be argued that iron incorporation into erythroblasts, shown to be impaired in ACD (Chapter 6), increases after L1 treatment.

BFUe colony counts were found to be reduced in ACD suggesting that the anemia has its basis at stem cell level (Chapter 9). This is probably indirectly mediated by increased RA activity. It is not known whether in ACD the erythroblasts become less sensitive to Epo which is added to BFUe mediums. If so, not only Epo responsiveness to the anemia is impaired but also Epo sensitivity of erythroblasts although of course other intrinsic erythroblast or medium factors could be involved. The low number of BFUe counts found in iron deficiency might be explained by low iron stores, available for erythroblast growth as well as RA disease activity.

We studied TNFα and IL-6 as possible mediators of impaired erythropoiesis in order to find a mechanism through which RA activity determines the anemia. Both hematopoietic growth factors probably play a pathogenetic role in RA [9,10]. Both serum TNFα and IL-6 correlated positively with parameters of RA activity like ESR and CRP. We investigated their effects on BFUe growth. TNFα was found to inhibit erythropoiesis in vitro whereas anti-TNFα resulted in a stimulation of erythropoiesis (Chapter 9). IL-6 in contrast had no consistent effects on erythropoiesis while anti-IL-6 exerted an inhibitory effect on BFUe

counts (Chapter 10). It was concluded that TNFα, its serum level being associated with RA activity is an important factor in ACD pathogenesis whereas IL-6 is not. Based on the findings of anti-IL-6 addition, IL-6 produced by monocytes in the medium might stimulate erythropoiesis and in this way counteract the effects of TNFα. It is obvious that in marrow cultures TNFα and IL-6 production takes place in vitro which complicates extrapolation of the findings to the in vivo situation.

In Table 1 study objectives of and results obtained by our investigations are shown.

TABLE 1 Study objectives of and results obtained by this thesis

STUDY OBJECTIVES	RESULTS OBTAINED
1 - Frequency of iron, B12 and folic acid deficiency in anemic RA patients. - Effects of these deficiencies on diagnostic parameters.	- 52%, 29% and 21% respectively. - Just one cause of anemia in only 24% of patients. - Normal values of cellular indices may mask combined deficiencies.
2 - Characterization of blood parameters in ACD with and without iron deficiency. - Indication of bone marrow aspiration.	- Reliable prediction of iron deficiency after adaption of reference values of MCV, ferritin and transferrin. - Bone marrow aspiration rarely indicated.
3 - Prediction of iron responsiveness of the anemia. - Increase of RA activity after iron treatment.	- Accurately, by means of MCV and serum ferritin after adapting their reference values. - No apparent serological effects.
4 - Role of impaired iron uptake and transferrin binding by erythroblasts.	- Iron incorporation and transferrin binding decreased in ACD. - Transferrin binding reduced in all RA patients. (Only significantly in ACD. - Overlap with iron deficiency due to RA activity levels comparable to ACD?

5 - Decreased Epo response to the anemia.	- Epo levels higher in RA patients compared to healthy controls. - Response insufficient to prevent anemia in ACD. - Possible negative correlation of serum Epo with iron stores.
6 - Effects of iron chelation on erythropoiesis and Epo responsiveness	- Hb and serum Epo rise - Possible correlation between Hb and Epo change. - Negative correlation of Hb and serum Epo change with ferritin change.
7 - The role of TNF-alpha in ACD	- BFUe numbers reduced in ACD - Increased serum TNF-alpha in ACD correlated with RA activity - Suppressive effects of TNF-alpha on in vitro erythropoiesis. - Stimulation of in vitro erythro-poiesis by anti-TNF-alpha addition addition.
8 - The role of IL-6 in ACD	- Increased serum IL-6 levels in ACD. - Positive correlation with RA activity. - Possible stimulatory effects on in vitro erythropoiesis. - Inhibitory effects of anti-IL-6 on in vitro erythropoiesis.

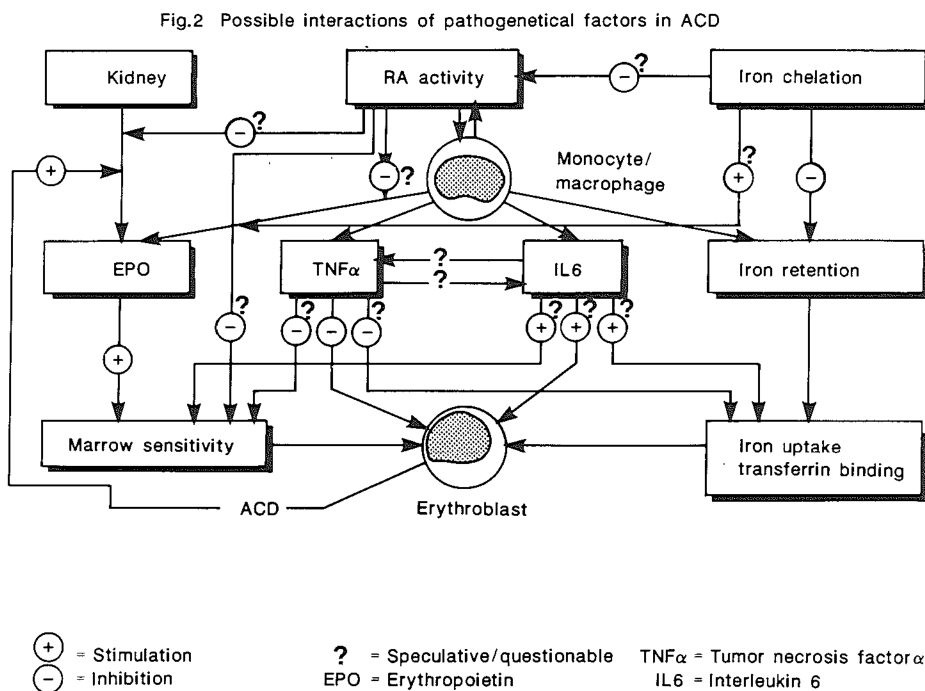
In conclusion, the present work shows that many potential treatable causes are present simultaneously in RA complicated by ACD. The finding of normal cellular indices may mask combined deficiencies whereas the presence of iron deficiency can be detected generally without performing a bone marrow aspiration for stainable iron assessment. Iron treatment is useful only in iron deficient RA patients and its response can easily be predicted by blood parameters.

The findings suggest that impaired erythropoiesis in ACD may be due to reduced iron uptake and transferrin binding by erythroblasts while Epo response to the anemia is decreased as well. Oral iron chelation by L1 might increase Hb through increased bone marrow iron availability or through a rise in Epo responsiveness after iron release from the MPS. TNFα, correlated with RA activity, might mediate impaired erythropoiesis while IL-6, correlated with RA activity as

well, might counteract the effects of TNFα.

It seems logical to assume that the factors associated with ACD studied here might interact with each other. Further studies are required to establish these interactions. In Figure 2 an effort is made to speculate on possible interrelationships.

FIGURE 2 Possible interactions of pathogenetical factors in ACD



CHAPTER 12

SAMENVATTING

In hoofstuk 1 werden de vele oorzaken van anemie bij RA alsmede de factoren geassocieerd met ACD beschreven. De diagnose ACD kan slechts per exclusionem gesteld worden. In veel van de beschreven studies bestaat er een onvoldoende classificatie van de ijzerstatus van de RA patiënten zodat bevindingen die in eerste instantie pasten bij ACD later ook bij ijzerdeficiëntie bleken voor te komen. Diverse mediatoren van erythropoiese werden besproken, doch deze stoffen zijn slechts in beperkte mate onderzocht als pathogenetische factor bij ACD.

De hoofddoelstellingen van dit proefschrift waren enerzijds het vaststellen van een betrouwbare methode om anemische RA patiënten te classificeren en anderzijds het nagaan of verminderde ijzerbeschikbaarheid voor erythroblasten, verminderde Epo respons op de anemie, en de effecten van TNFα en IL-6 op de (in vitro) erythropoiese een rol spelen in de pathogenese van ACD.

In hoofdstuk 2 werden de hematologische en klinisch chemische bepalingen en de methoden met betrekking tot de in vitro experimenten met erythroblasten alsmede de statistische methoden beschreven.

In hoofdstuk 3 werd aangetoond dat slechts 24% van de anemische patiënten één oorzaak van anemie hebben (ACD). De overigen hebben combinaties van deficiënties (ijzer, B12 en foliumzuur) en ACD die gemaskeerd kunnen worden door het vinden van normale indices.

In hoofdstuk 4 werd verder ingegaan op de ijzerstatus van de RA patiënten die in 52% van de gevallen ijzerdeficiënt bleken te zijn. Indien de referentiewaarden van MCV, ferritine en transferrine enigzins aangepast worden, voorspellen zij nauwkeurig de aanwezigheid van ijzerdeficiëntie. Een beenmergaspiratie voor kleurbaar ijzer is derhalve zelden geïndiceerd.

In hoofdstuk 5 bleek dat dezelfde parameters, met name MCV en ferritine ook een Hb stijging na ijzerbehandeling nauwkeurig konden voorspellen. Alleen ijzerdeficiënte RA patiënten hadden een Hb stijging. Blijkbaar leidt het toedienen van extra ijzer niet tot verhoogde ijzerbeschikbaarheid voor het beenmerg bij ACD patiënten.

Deze verminderde ijzerbeschikbaarheid is verder onderzocht in hoofdstuk 6. Bij RA patiënten met ACD werd een evident verminderde

ijzerincorporatie in de erythroblasten gevonden die correleerde met de RA activiteit. Bij ACD is de transferrine binding aan de erythroblast ook verminderd hetgeen de lagere ijzerincorporatie verklaart, hoewel er overlap was met de ijzerdeficiënte groep waarschijnlijk als gevolg van overlap in RA activiteit. Het lijkt dat verminderd ijzertransport naar de erythroblast een rol zou kunnen spelen als pathogenetische factor bij ACD.

In hoofdstuk 7 deel 1 en 2 werd beschreven dat serum Epo spiegels hoger zijn in alle RA groepen in vergelijking met controles. Bij ACD lijkt de Epo respons iets minder dan bij ijzerdeficiëntie en is dus onvoldoende om het Hb-niveau te handhaven. Er werd een negatieve correlatie tussen ferritine -als maat voor de ijzervoorraad- en serum Epo gevonden.

Dit mogelijk negatieve verband werd vervolgens in hoofdstuk 8 verder nagegaan door 10 RA patiënten met ACD te behandelen met de orale ijzerchelator L1. Het ijzer werd effectief uit de ijzervoorraad gecheleerd en een Hb en Epo stijging werd gevonden waarbij de Hb en Epo veranderingen positief correleerden. Bovendien correleerden de Hb en Epo veranderingen negatief met ferritine veranderingen. Het lijkt derhalve mogelijk dat de Epo respons bij ACD toeneemt na ijzerafgifte van het MPS. De Hb stijging zou dus verklaard kunnen worden door de toegenomen Epo respons of door een verhoogde ijzerbeschikbaarheid voor erythroblasten door ijzeruitwisseling tussen transferrine en ferritine. De resultaten zijn echter preliminair.

Hoofdstuk 9 en 10 beoordelen de rol van de cytokines TNFα en IL-6 bij ACD. In vitro bleek de erythroïde groei (aantal BFUe kolonies) geremd te zijn. Ook bij ijzerdeficiëntie bleek dit het geval te zijn, hetgeen verklaard werd door verminderde ijzervoorraden beschikbaar voor erythroblasten, en ziekteaktiviteit. Gezien de positieve correlatie van serum TNFα en IL-6 met RA ontstekingsparameters alsmede het feit dat de spiegels het hoogst waren in de ACD groep werden de effecten van deze cytokines op de erythroïde groei in vitro onderzocht. TNFα bleek suppressie te geven van de erythroïde groei terwijl anti-TNFα stimulatie gaf. IL-6 had inconsistente effecten op het aantal BFUe kolonies en anti-IL-6 gaf inhibitie. Het zou dus mogelijk kunnen zijn dat TNFα, verhoogd bij actieve RA, een oorzaak is van ACD terwijl IL-6, gezien de negatieve effecten van anti-IL-6,

juist deze remming tegengaat. Waarschijnlijk is er veel TNFα en IL-6 productie in het medium waardoor bijvoorbeeld de stimulerende effecten van IL-6 gemaskeerd werden. Diezelfde in vitro cytokine productie maakt extrapolatie van de in vitro effecten naar de in vivo situatie echter moeilijk.

In Figuur 2 van hoofdstuk 11 wordt een schema voorgesteld voor de mogelijke samenhang van de diverse pathogenetische factoren met betrekking tot ACD zoals in dit proefschrift bestudeerd.

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CHAPTER 11

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 27 januari 1959 te Rotterdam geboren. Na het behalen van het Atheneum B diploma aan het Develsteincollege te Zwijndrecht studeerde hij van 1977 tot 1978 Economie aan de Erasmus Universiteit Rotterdam, waarna hij in 1978 begon met de studie Geneeskunde, eveneens aan de Erasmus Universiteit te Rotterdam. Op 21 december 1985 behaalde hij het artsexamen.
Gedurende zijn studie was hij achtereenvolgens als student-assistent verbonden aan de vakgroepen Pathologische Anatomie II (Hoofd: prof. Dr. M. de Vries), Inwendige Geneeskunde II (Hoofd: Prof. J.H.P. Wilson, begeleider: Prof. Dr. S.W. Schalm) en Kindergeneeskunde (Hoofd: Prof. Dr. H.K.A. Visser, begeleider: Dr. M. Sinaasappel).
Van 1 januari tot 30 juni 1986 was hij als AGNIO werkzaam op de afdeling Inwendige Geneeskunde van het Catharina Ziekenhuis te Eindhoven (Opleider: Dr. H.F.P. Hillen), waarna hij op 1 juli 1986 met zijn opleiding tot internist begon op de afdeling Inwendige Geneeskunde van het Zuiderziekenhuis te Rotterdam (Opleider: Dr. P.W. de Leeuw), in het kader waarvan hij van 1 maart 1988 tot 28 februari 1989 als stagiair werkzaam was op de afdeling Rheumatologie van de Dr. Daniel den Hoed Kliniek (Hoofd: Dr. A.J.G. Swaak).

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