# OXYGEN FREE RADICALS IN RHEUMATOID ARTHRITIS

## VRIJE ZUURSTOF RADICALEN IN RHEUMATOIDE ARTHRITIS

**PROEFSCHRIFT**

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 12 NOVEMBER 1986 TE 15.45 UUR

DOOR

**PIETER BIEMOND**

GEBOREN TE ZEVENHUIZEN

1986

OFFSETDRUKKERIJ KANTERS B.V.,
ALBLASSERDAM

Promotiecommissie:

Promotor : Prof. Dr. J.F. Koster

Overige leden: Prof. Dr. J.C. Birkenhäger

Prof. Dr. H.G. van Eijk

Prof. Dr. L.B.A. van de Putte

The cover shows a drawing from Hans Boomsma, who suffers from rheumatoid arthritis.

Financial support of Pfizer B.V. is gratefully acknowledged.

Aan: Corry

Christiaan, Liesbeth en Benjamin

CONTENTS

VOORWOORD 7

CHAPTER 1.

INTRODUCTION 9

General information on rheumatoid arthritis 10

Clinical aspects of rheumatoid arthritis

Etiology of rheumatoid arthritis

Pathogenesis of rheumatoid arthritis

Iron metabolism in rheumatoid arthritis

Oxygen free radicals in biology 16

The nature of oxygen free radicals

Sources of oxygen free radicals

Interaction between iron and oxygen free radicals

Biological effects

Protectors

Oxygen free radicals in ischemia

Evidence for a role of oxygen free radicals in the pathogenesis of rheumatoid arthritis 23

Sources of oxygen free radicals in rheumatoid arthritis

Detection of oxygen free radical dependent tissue damage

Beneficial effect of oxygen free radical protectors in rheumatoid arthritis

The aim of the study 25

CHAPTER 2.

Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid 27
(Arthritis Rheum. 1984, 27:760-765)

CHAPTER 3.

Intracellular and extracellular sulfhydryl levels in rheumatoid arthritis 41
(Ann. Rheum. Dis. 1986, 45:44-46)

CHAPTER 4.

Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leucocytes. Possible mechanism in inflammation diseases 49
(J. Clin. Invest. 1984, 73:1576-1579)

CHAPTER 5.

Intra-articular ferritin bound iron in rheumatoid arthritis 61
A factor multiplicating oxygen free radical induced tissue destruction
(Arthritis Rheum., in press)

CHAPTER 6.

On the superoxide dependent and independent mechanism of iron mobilization from ferritin by xanthine oxidase. Its implications for oxygen free radical induced tissue destruction during ischemia and inflammation 77
(Biochem. J., in press)

CHAPTER 7.

Superoxide production by polymorphonuclear leucocytes in rheumatoid arthritis and osteoarthritis: In vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH-oxidase 91
(Ann. Rheum. Dis. 1986, 45:249-255)

CHAPTER 8.

GENERAL DISCUSSION 105

Protective factors against oxygen free radicals in rheumatoid arthritis
Availability of iron able to catalyse the formation of hydroxyl radicals
Hydroxyl radical formation in ischemia
Indication of iron administration in rheumatoid arthritis
Inhibition of superoxide production from granulocytes by piroxicam
Outlook

CHAPTER 9.

SUMMARY 115

CHAPTER 10.

POPULAIRE SAMENVATTING 117

Reuma

Bescherming tegen giftige zuurstofprodukten

Toename van beschadiging door giftige zuurstofprodukten onder invloed van ijzer

Bestrijding van het ongunstige effect van ijzer

De invloed van bekende geneesmiddelen tegen reuma op giftige zuurstofprodukten

Conclusie

GENERAL REFERENCES 121

OVERIGE PUBLICATIES 129

CURRICULUM VITAE 131

VOORWOORD

Dit proefschrift werd bewerkt op de afdeling Biochemie I van de Erasmus Universiteit Rotterdam. De volgende mensen wil ik met name bedanken voor hun hulp: Mijn promotor Hans Koster, die mij de ruimte gaf en stimuleerde door leerzame discussies. Het was altijd gezellig om bij je langs te lopen, al was het maar vanwege jouw vermogen om alles, dit onderzoek maar ook het universitaire en medische gebeuren, te relativeren. De leden van de promotiecommissie dank ik voor de vlotte beoordeling: Prof. Dr. J.C. Birkenhäger, die ik tevens wil bedanken voor de ruimte die hij gaf om mijn proefschrift rustig af te ronden; Prof. Dr. H.G. van Eijk, ook voor de nuttige discussies over ijzer metabolisme die gedurende het onderzoek plaatsvonden en Prof. Dr. L.B.A. van de Putte, die als rheumatoloog bereid was het proefschrift te beoordelen. Tom Swaak, die mij mede heeft begeleid, is één van de weinige perifeer werkzame specialisten die een wezenlijke bijdrage aan wetenschappelijk onderzoek leveren. Jouw stortvloed van ideeën bevatte altijd bruikbare suggesties. Regina Slee, die mij heeft laten profiteren van haar perfectionisme bij het uitvoeren van experimenten. Christiaan Beindorff, die gedurende de tweede helft van het onderzoek al het praktische werk heeft verricht en om 8 uur present was, zodat wij voor de start van mijn klinische werk de lopende zaken door konden praten. De praktische hulp van Wim van Noort en Martin Kroos was zeer welkom. Dr. J.R.W. ten Kate en Dr. M.P. Heyboer voor de medewerking bij het verkrijgen van materiaal van patiënten met traumatische knie letsels. Martha Wieriks die in korte tijd het manuscript klaar maakte voor de drukker. Hiernaast alle overige mensen van de afdeling Biochemie I die op diverse manieren mij bijgestaan hebben en menigmaal hun bloed voor mijn proeven hebben laten vloeien. Corry, jou wil ik bedanken voor je morele steun en dat je het grootste deel van de verzorging van onze kinderen op je hebt genomen, zodat ik vooral van hen kon genieten.

# CHAPTER 1.

# INTRODUCTION

## GENERAL INFORMATION ON RHEUMATOID ARTHRITIS

### CLINICAL ASPECTS OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) can be described briefly as a chronic, symmetric, erosive polyarthritis. The diagnosis RA is based on a couple of criteria (table 1). The American Rheumatism Association criteria are most frequently used (1). "Classical" RA needs 7 criteria, if only five criteria are met it is called "definite" RA.

The mean symptom of RA is the articular inflammatory reaction. Although every joint can be involved, some are more frequently affected, like the metocarpophalangeal joints, while others are relatively spared like the distal interphalangeal joints. Typically the inflammation is symmetrically distributed, causes bone erosions and becomes chronic. The inflammatory process results in thickening of the synovial membrane, and increase in the amount of synovial fluid with decreased viscosity. After some time bone erosions will occur, cartilage is lost and total destruction of the joint, with complete functional loss can be the final result.

Rheumatoid arthritis is not limited to joints but a variety of extra-articular manifestations show that it is a systemic disorder (2):

- rheumatic nodules
- vasculitis at various locations
- pericarditis
- pleurisy, involvement of lung parenchyma
- keratoconjunctivitis sicca
- neuropathy
- lymphadenopathy
- splenomegaly
- neutropenia

It is uncertain whether RA is one disease with variable expression, or that in fact RA encloses several different diseases. Ankylosing spondylitis is described in the older literature as a particular form of RA. By now it is generally accepted to be a separate disease entity. Possibly, in the future also other diseases will be separated from what is currently called RA.

Rheumatoid arthritis is a common disorder. Epidemiologic studies reveal a prevalence rate of classical and definite RA between 0.3 and 1.5 percent (3). It is about three times more common in females than in males, and the beginning is most frequently between 40 and 60 years of age.

A typical feature of RA is the presence of auto-antibodies, directed against a variety of structures, for example: antibodies against cartilage, anti-perinuclear factors, and anti-nuclear factors. About 70% of the RA patients have antibodies directed against the Fc-fragment of immunoglobulin G, which are called rheumatoid factors.

Table 1. ARA-criteria for the diagnosis of RA.

1. Morning stiffness.
2. Pain in one joint.
3. Swelling in one joint.
4. Swelling of a second joint.
5. Symmetry in joint swelling.

To meet criteria 1-5 the symptoms must be present for at least six weeks.

6. Subcutaneous nodules.
7. Periarticular bone erosions.
8. Presence of rheumatoid factor.
9. Synovial fluid with decreased viscosity.
10. Synovial membrane histopathology consistent with RA:
    a. villous hypertrophy.
    b. synovial cell proliferation.
    c. mononuclear infiltration.
    d. fibrin deposition.
11. Typical histopathology of rheumatic nodules.

In addition it has to be excluded that the clinical picture is caused by other diseases like: systemic lupus erythematodus, gout, scleroderma or ankylosing spondylitis.

## ETIOLOGY OF RHEUMATOID ARTHRITIS

The etiology of RA is unknown, despite the enormous amount of data which are available. The interaction between multiple etiological factors most likely results in the development of RA. The following factors seem to be essential:

- genetic predisposition
- initiating factor
- immunodisregulation

### Genetic predisposition

Although the data of different studies are variable, an increased RA frequency is found in relatives of RA patients. The presence of antigens of the major histocompatibility complex are genetically determined. The HLA-DR 4 antigen is found more frequently in RA patients compared to controls (4, 5). It is suggested that genes coding for this HLA antigen are associated with genes regulating immune response, which determine an immunological response that results in RA (6).

### Initiating factor

An enormous amount of initiating factors are suggested in RA. Many studies are unreliable due to lack of an appropriate control group. A lot of potential initiating factors are also found in other types of arthritis and are likely to be only secondary phenomena. However, it is possible that one or more of them are important in the etiology of RA. Potential initiating factors can be divided in exogenous and endogenous factors:

- Exogenous factors

A couple of different bacteria and viruses are nominated. Clostridium perfringens, especially atypical forms are frequently found in faeces of RA patients (7). Diphteroids were isolated in 28% from synovial membrane in RA (8). Mycoplasmata can produce a disease in animals resembling RA (9) and some investigators were able to isolate the organism from RA joints.

From the viral candidates the Epstein-Barr virus is especially interesting. Antibodies are detected in RA directed against an antigen found on cells infected with this virus (10). The virus is known to

cause polyclonal B-cell stimulation, revealing increased immunoglobulin production. The same modulation of the immune system does occur in RA. It is possible that by this mechanism the Epstein-Barr virus facilitates the development of RA (11).

- Endogenous factors

Collagen is an example of an endogenous factor which might be important. Antibodies to different types of collagen were found in RA (12) and injection of type II collagen in rats resulted in chronic arthritis (13).

Immunoglobulin G (IgG) is possibly a factor of critical importance. Antibodies to IgG, called rheumatoid factors, are present in serum of about 70% of RA patients, where it is accompanied by more severe clinical disease (14). In the synovium of RA patients the rheumatoid factor is synthesized in signficant amounts, sometimes the rheumatoid factor even can be found in the synovial fluid while it is not present in the serum (15, 16). Rheumatoid factor can form immune complexes, able to activate complement and to stimulate granulocytes resulting in an inflammatory response. It is possible that the rheumatoid factor is essential in the etiology of seropositive RA, and that seronegative RA in fact is a different disease. The production of antibodies directed against IgG can be explained by two hypothetical mechanisms:

a. An inbalance between T-helper and T-suppressor lymphocytes allow the production of antibodies to native IgG. This disorder in immunoregulation might be genetically determined or acquired.
b. The IgG molecule is changed, for example by proteases or by oxygen free radicals, so new antigenic determinants are presented, and antibody production follows.

Immunodisregulation

HLA-DR 4 antigens, which are frequently found in RA are closely related to immune response genes (17). Many alterations of immunocompetent cells are suggested in RA. A considerable part might be only secondary phenomena. However, it is likely that the balance between T-helper and T-suppressor cells is disturbed, resulting in abnormal antibody production and an ongoing cellular inflammatory response.

Further investigations have to show which factors are important in the etiology of RA and how they interact. Again the possibility has

to be considered that RA consists of a group of two or more different diseases.

## PATHOGENESIS OF RHEUMATOID ARTHRITIS

In contrast to the etiology, much more is known about the pathogenesis of RA. The main features of the pathophysiological mechanism are shown in figure 1. The events are artificially divided into four parts:

A: The unknown antigenic stimulus, which is discussed in the previous section, will cause an immune response.

B: The immune response will cause the formation of immune complexes containing rheumatoid factor and other auto-antibodies. In addition a variety of lymphokines like interleukin I and growth factors are formed.

C: The immunecomplexes in synovial fluid are phagocytosed by polymorphonuclear leucocytes (PMN), the dominant inflammatory cell within the joint fluid. The stimulated PMN produce hydrolytic enzymes, leucotrienes, prostaglandins and oxygen free radicals, which cause tissue damage on their own, and also activate the complement, clotting and kinin systems.

D: In the synovial membrane the inflammatory mediators cause a chronic inflammation in which lymphocytes and macrophages play a dominant role. The synovial membrane proliferates and an invasive front is formed, called the pannus, which extends on the surface of the cartilage. Pannus cells are attracted by immune complexes which diffuse from the synovial fluid into the cartilage. The proliferating cells produce different enzymes like cathepsins and elastase, and prostaglandins able to destruct proteoglycans and collagen.

Finally the cartilage is damaged by inflammatory mediators produced by both the synovial membrane including the pannus, and by PMN from the synovial fluid. In addition mineral is resorbed from bone by osteoclasts activated by prostaglandins and other mediators.

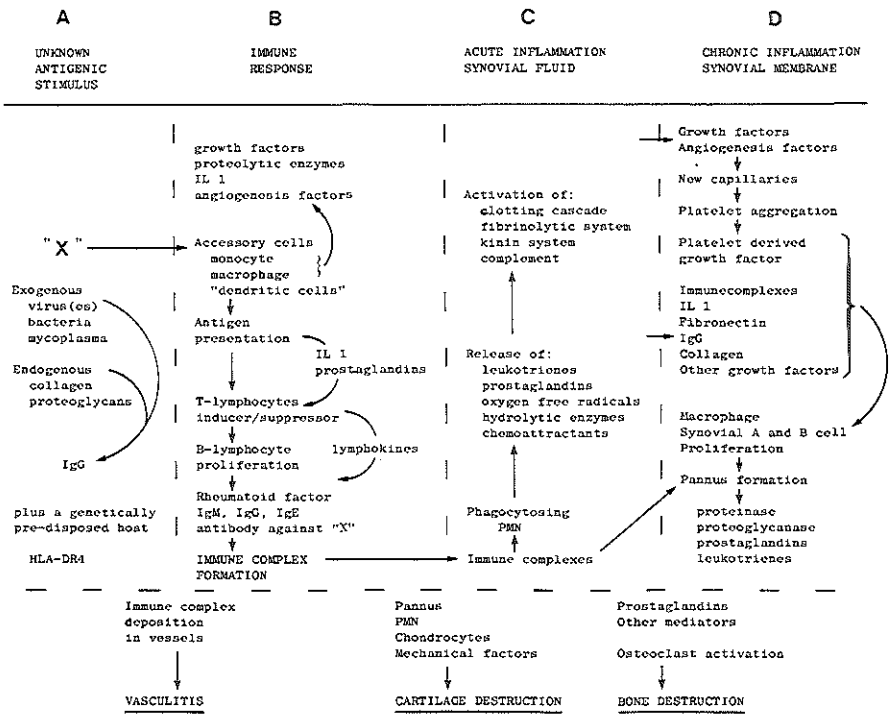



Fig.1: The pathogenesis of rheumatoid arthritis. (modified from ref 2)

## IRON METABOLISM IN RHEUMATOID ARTHRITIS

Iron plays an important role in the toxicity of oxygen free radicals, which will be discussed in the next chapter. Therefore it is important to consider the alteration in iron metabolism found in RA. Anemia is almost invariably found in RA, and its severity is related to the activity of the inflammation. This type of anemia is also found in other inflammatory disorders and in cancer, in fact it can be found in all patients which are seriously ill, therefore it has been called the anemia of chronic diseases. Characteristically the serum iron concentration is depressed. However, in contrast to real iron deficiency, administration of iron is ineffective. Most likely the low serum iron concentration is caused by an inability to mobilize iron from its stores. The largest stores are macrophages, for example in

the spleen, and liver parenchymal cells. In most RA patients iron is present in the bone marrow, but it is stored in macrophages, and the mobilisation is disturbed. So no iron is available for hemoglobin synthesis in erythroid cells resulting in anemia. Interleukin II is suggested to be the factor responsible for the defective iron mobilization (18). The distribution of iron is severely changed in RA, in serum it is decreased, but in the synovial membrane it is strongly elevated (19).

## OXYGEN FREE RADICALS IN BIOLOGY

### THE NATURE OF OXYGEN FREE RADICALS

Free radicals are molecules which contain an atom with an unpaired electron in its outer orbital. Radicals are very reactive in order to restore the situation of paired electrons, resulting in a lower energy state. Molecular oxygen ($O_2$) is in fact a bi-radical because it contains two electrons in the outer orbital with the same spin direction. This explains the relative high reactivity of oxygen. If radicals are formed, they are likely to react with oxygen, because the universal availability of oxygen and its reactivity, with the result that most radicals in the biological systems contain oxygen.

In normal aerobic metabolism, oxygen is reduced in a tetravalent way, by the final part of the respiratory chain, cytochrome $aa_3$. On the other hand univalent reduction of oxygen, which can occur in many different circumstances, will reveal superoxide ($O_2^-$) and the extremely reactive hydroxyl radical ($OH^\cdot$) (figure 2).

TETRAVALENT OXYGEN REDUCTION

$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$

UNIVALENT OXYGEN REDUCTION

| | | |
|---|---|---|
| $O_2 + e^- \rightarrow$ | $O_2^-$ | (Superoxide) |
| $O_2^- + e^- + 2H^+ \rightarrow$ | $H_2O_2$ | (Hydrogen Peroxide) |
| $H_2O_2 + e^- + H^+ \rightarrow$ | $H_2O + OH^\cdot$ | (Hydroxyl Radical) |
| $OH^\cdot + e^- + H^+ \rightarrow$ | $H_2O$ | |

Fig.2: Free radical production during oxygen reduction.

SOURCES OF OXYGEN FREE RADICALS

Both endogenous and exogenous sources of oxygen free radicals are identified (20).

Endogenous sources

a. The mitochondrial electron transport chain always produces small quantities of oxygen free radicals (21), the amount will increase when only a small amount of oxygen is available, because reduction of all respiratory chain carriers will result in univalent reduction of oxygen.
b. Microsomes contain several enzyme systems, for example cytochrome $P_{450}$, able to produce radicals.
c. Soluble enzymes like xanthine oxidase.
d. Macrophages and PMN are able to produce superoxide (22). After stimulation of these cells, a membrane bound NADPH-oxidase complex is activated, resulting in production of superoxide within the phagocytosing vacuole and on the outer surface of the cells (figure 3). The production of superoxide is important in the killing of phagocytosed bacteria. The importance is illustrated by the fact that patients with chronic granulomatous disease, a disease characterised by an inability of PMN to produce superoxide, usually die due to infections. On the other hand, superoxide is released extracellularly, which can seriously damage the surrounding tissues.

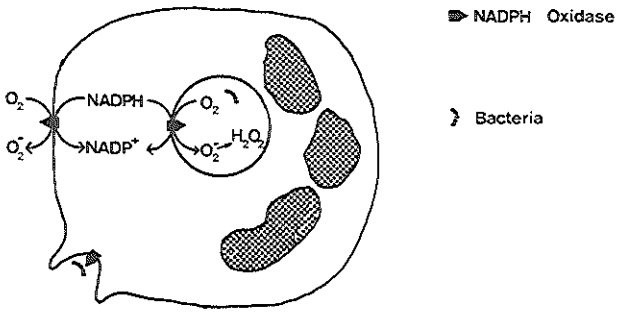



Fig. 3: Superoxide production by polymorphonuclear leucocytes.

Exogenous sources

Radiation of different wave-length causes damage due to free radical formation in tissues. The destructive effects of many toxic chemicals depends on free radical reactions (23). The effects, and side-effects of several chemotherapeutic agents, like doxorubicin and bleomycin, are radical mediated (24).

## INTERACTION BETWEEN IRON AND OXYGEN FREE RADICALS

### Conversion of oxygen free radicals by iron.

Although superoxide is not completely innocent, its toxicity does strongly increase in the presence of iron. Superoxide ($O_2^-$) can be converted to the extremely reactive hydroxyl radical ($OH^\cdot$) in the Haber-Weiss reaction (25) (reaction a). However, this reaction is energetically impossible under normal conditions.

a. $O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH^\cdot$

Iron is able to catalyse this reaction as shown in reaction b and c.

b. $O_2^- + Fe^{+++} \longrightarrow O_2 + Fe^{++}$

c. $Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^- + OH^\cdot$

Additionally iron can increase oxygen free radical damage by the formation of iron-oxygen radicals with increased reactivity, for example perferryl and ferryl radicals (26).

### The availability of iron

Non-protein bound iron is able to catalyse hydroxyl radical formation, but it is not present in biological systems. Nearly all the body iron is located in haem-containing proteins, and in iron-binding proteins such as transferrin, ferritin, lactoferrin, or in enzymes. The question wether protein-bound iron is also able to stimulate the formation of more reactive radical species, has been subject of much research. Many investigations in vitro show that protein-bound iron does not catalyse $OH^\cdot$ formation (27, 28, 29). Furthermore, prevention of $OH^\cdot$ production by lactoferrin and transferrin was found, based on the binding of free iron present in the test system (30, 31). In

contrast, other studies revealed that iron-binding proteins were able to stimulate $OH^{\cdot}$ formation (32, 33, 34, 35). In these studies, however, the iron binding proteins were fully saturated with iron, as far as the iron saturation was mentioned, a situation which does seldom occur in vivo. Baldwin et al. (36) probably solved the problem by showing that neither transferrin nor lactoferrin stimulated $OH^{\cdot}$ formation, and explained other results by free iron present in the materials used.

Ferritin requires some special attention, for different groups found that it could stimulate $OH^{\cdot}$ formation (37, 38, 39), although Gutteridge et al. (29) found no effect. The mechanism by which ferritin did increase oxygen free radical damage was unknown. In this thesis new information about the role of ferritin is presented.

## BIOLOGICAL EFFECTS

The destructive effects of oxygen free radicals are based on their reactions with a wide spectrum of biomolecules. Freeman et al. (20) recently reviewed these targets.

Major targets are:

- Proteins

Unsaturated or sulfur containing aminoacids are especially sensitive for free radical attack. This will result in structural alterations of proteins and loss of their function.

- Nucleic acids

Strand scission and base modification are known to occur. These events are related to mutations and malignant transformation of cells.

- Lipids

Membranes are major targets for oxygen free radical damage, firstly because oxygen is five times more soluble in lipids compared to water (40) and secondly because polyunsaturated fatty acids are very sensitive to free radical attack. The process of the breakdown by oxygen free radicals is called lipid peroxidation. One initiating radical can cause a chain of reactions, breaking down the fatty acid, producing a high amount of secondary radicals and many breakdown products, for example malondialdehyde, which is often used to measure lipid peroxidation, and hydroxynonenal. Many of these intermediates possess in-

trinsic toxic effects.

- Carbohydrates

Resulting in changes in cell surface receptors and glycoproteins.

- Hyaluronic acid

This macromolecule is important to maintain the high viscosity of synovial fluid. It is depolymerised by oxygen free radicals resulting in a decrease in viscosity.

- Collagen

Interference with the polymerisation of collagen occurs.

- Proteoglycans

Destruction of proteoglycans and collagen will interfere with the normal structure of cartilage.

It is clear that if oxygen free radicals are produced in a biological system, damage will occur. Membranes desintegrate due to lipid peroxidation leading to cell death, metabolic pathways are blocked due to enzyme destruction, structural molecules are damaged, and so on. In fact the human being can survive not because the presence of oxygen but despite the presence of oxygen. For survival a complex of many protectors is necessary.

On the other hand, higher organisms have learned to use their enemy: granulocytes and macrophages produce superoxide to kill phagocytosed mirco-organisms (22, figure 3).

## PROTECTORS

In life well-being often depends on a balance between good and evil. This general statement is also true with regard to oxygen free radicals. The occurrence of damage due to oxygen free radicals depends on the balance between the amount and type of radicals which are formed, and the amount of protectors present at the same location (figure 4).

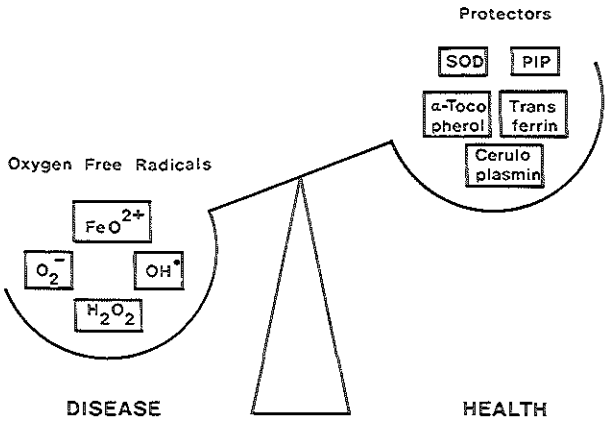



Fig. 4: The balance between oxygen free radicals and protectors.

Enzymatic protection

Protection against oxygen free radicals depends partially on specific enzymes. Superoxide dismutase (SOD) is able to convert $O_2^-$ to $H_2O_2$. Catalase and glutathione peroxidase complete the detoxification by further reduction of $H_2O_2$ to $H_2O$ (figure 5). In contrast to catalase glutathione peroxidase is able to convert peroxidated polyunsaturated fatty acids to the hydroxy-form. Peroxidated fatty acids in lipids cannot be reached by glutathione peroxidase. Recently an enzyme is discovered, called peroxidation inhibiting protein (PIP), which is able to react with peroxidated fatty acids in phospholipids, yielding the corresponding alkoholic fatty acid (41).

Both glutathione peroxidase and PIP need reduced glutathione (GSH) as electron donor. GSH is recovered from oxidised glutathione (GSSG) by glutathione reductase, consuming NADPH formed in the pentose shunt. All these protective enzymes are present almost exclusively intracellularly.

Non-enzymatic protection

A large group of scavengers are available protecting by non-enzymatic mechanisms. Some important representatives are shown in figure 5. Vitamin E (α-Tocopherol) is important in the protection of membranes against lipid peroxidation. GSH is essential as co-factor for glutathione peroxidase and PIP. Extracellular fluids contain only

minor amounts of scavengers. Ceruloplasmin is the major protector in body fluids. Interestingly, it is an acute phase reactant increasing in concentration during active inflammation. This seems to be a compensatory mechanism for the oxygen radicals produced during inflammation. Iron-binding proteins can protect by binding iron and so prevent the formation of more destructive oxygen free radicals, as described previously.

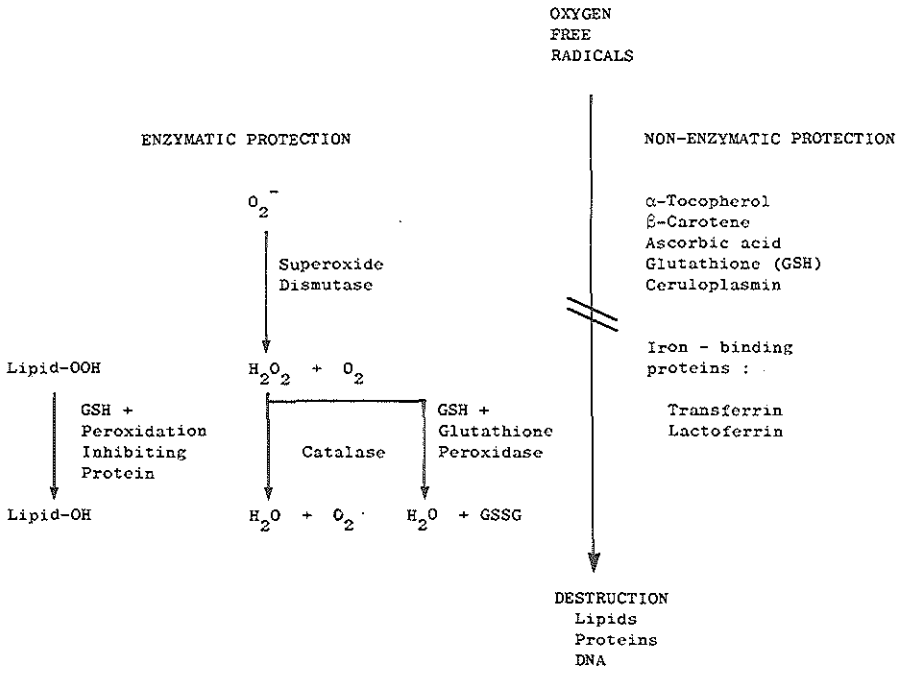



Fig.5: Protectors against oxygen free radicals.

## OXYGEN FREE RADICALS IN ISCHEMIA

Many pathological events are related to oxygen free radical damage: a wide spectrum of inflammatory diseases (42, 43, 44), direct and late tissue destruction by irradiaton (45, 46), toxicity of many chemicals (23), the effect of high oxygen pressure on lungs (47, 48), and the effects and side-effects of cytostatic drugs like the antrocyclins (24).

The important role of free radicals in the pathogenesis of tissue damage in ischemia is increasingly recognised. In physiological circumstances xanthine dehydrogenase is present in many tissues, converting xanthine to uric acid, using $NAD^+$ as electron acceptor. During ischemia xanthine dehydrogenase is converted to xanthine oxidase which uses $O_2$ as electron acceptor, leading to the production of $O_2^-$ and $H_2O_2$ (42, 49). Simultaneously in ischemia ATP is broken down via AMP to xanthine, which together with oxygen, present due to reperfusion or due to partial ischemia, are the substrates necessary for the production of oxygen free radicals. This hypothesis is supported by the beneficial effects of superoxide dismutase during intestinal and myocardial ischemia (50, 51). In addition post-ischemic tissue destruction could be inhibited by allopurinol, an inhibitor of xanthine oxidase (50-54).

# EVIDENCE FOR A ROLE OF OXYGEN FREE RADICALS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

## SOURCES OF OXYGEN FREE RADICALS IN RHEUMATOID ARTHRITIS

Quantitatively the most important sources of oxygen free radicals in RA are the PMN in synovial fluid and macrophage-like cells in the synovial membrane. Up to 70% of the synovial lining cells are type A cells, which are derived from blood monocytes, and in the synovial fluid in RA the number of PMN can increase to 75 x $10^9$/l. Macrophages and PMN will phagocytose immunecomplexes, cellular debris and other substances. Active phagocytosis is accompanied by activation of the

membrane bound NADPH-oxidase complex, which will release $O_2^-$ in the phagocytosing vacuole and outside the cell (22, figure 3). An additional source of oxygen free radicals might be xanthine oxidase formed during relative ischemia (55) or due to proteolytic attack on xanthine dehydrogenase.

## DETECTION OF OXYGEN FREE RADICAL DEPENDENT TISSUE DAMAGE

Direct measurement of oxygen free radicals in biological materials are impossible because of their very short half-life. So only the detection of products, which are typical for free radical attack, can be used. Malondialdehyde is found in serum and synovial fluid in RA (56, 57). It has to be realised, however, that malondialdehyde is not only formed during lipid peroxidation but also during prostaglandin synthesis. Lipid peroxidation products of higher molecular weight are detected in synovial fluid of RA patients (58, 59), providing stronger evidence for oxygen free radical reactions.

Hyaluronic acid, a glycosaminoglycan, present in normal synovial fluid has a very high molecular weight. This is responsible for the high viscosity of synovial fluid making it a perfect lubricant. However, in RA hyaluronic acid is depolymerised and the viscosity is decreased to the value of serum. How to explain the depolymerisation? In synovial fluid no hyaluronidase is present and the proteases formed by granulocytes are unable to degrade hyaluronic acid. It is evident that hyaluronic acid is very sensitive to oxygen free radical attack, resulting in its depolymerisation (60). It can be concluded that the presence of low molecular weight hyaluronic acid in RA is caused by free radical attack.

## BENEFICIAL EFFECTS OF OXYGEN FREE RADICAL PROTECTORS IN RHEUMATHOID ARTHRITIS

In controlled trials SOD is shown to be effective in RA (61, 62). However, the results of these studies are variable and no placebo is used in the control group. It is hard to believe that injection of SOD a few times a week is adequate to destroy all the superoxide which is formed, in view of the very short half-life of SOD (approximately 20

min.). More studies are needed to evaluate the usefullness of SOD in RA.

Drugs which are commonly used in RA are tested for their effects on oxygen free radicals. D-penicillamin, especially its copper conjugate possess SOD activity (63). Gold compounds and a spectrum of non-steroidal anti-inflammatory drugs (NSAID) are found to inhibit superoxide production by granulocytes in vitro (64-66).

## THE AIM OF THE STUDY

Current knowledge strongly suggests that oxygen free radicals are involved in the pathogenesis of RA. Additional information about the mechanism of free radical attack is necessary in order to find out if interaction with the mechanism of free radical damage can be used in the treatment of this crippling disease.

Attention is focussed on the following main questions:

- Are protective factors against oxygen free radicals present in sufficient amounts in RA (chapter 2, 3).
- Is iron available in RA in a form able to catalyse the formation of hydroxyl radicals (chapter 4-6).
- Are non-steroidal anti-inflammatory drugs able to inhibit $O_2^-$ production in vivo (chapter 7).

CHAPTER 2.

# PROTECTIVE FACTORS AGAINST OXYGEN FREE RADICALS AND HYDROGEN PEROXIDE IN RHEUMATOID ARTHRITIS SYNOVIAL FLUID

P. Biemond, A.J.G. Swaak and J.F. Koster

Arthritis Rheum 27:760, 1984

SUMMARY

Oxygen free radicals are probably involved in the pathogenesis of rheumatoid arthritis (RA). The enzymes involved in protection against oxygen free radicals and $H_2O_2$ (superoxide dismutase, catalase and glutathione peroxidase) were measured. Superoxide dismutase was not increased, glutathione peroxidase was slightly and catalase was strongly elevated in RA synovial fluid (SF) compared with control SF. Although these enzymes are present in SF, the activities are insufficient to protect against oxygen free radicals and $H_2O_2$. In contrast to transferrin, ferritin was increased in RA synovial fluid. Ceruloplasmin was also elevated. When rat liver microsomes were used as a target for oxygen free radicals, serum and SF were both protective. Gel filtration experiments showed that the fraction pattern in which there was maximal protective potential against lipid peroxidation corresponded closely to the level of ceruloplasmin. After removal of ceruloplasmin from serum or SF, about 70% of the protective capacity disappeared. It is concluded that ceruloplasmin is an important protector against oxygen free radicals.

INTRODUCTION

Evidence is accumulating which suggests that oxygen free radicals such as superoxide ($O_2^-$) and hydroxyl radical ($OH^\cdot$), and related oxygen species such as hydrogen peroxide ($H_2O_2$) and singlet oxygen, are involved in the pathogenesis of rheumatoid arthritis (RA). During phagocytosis granulocytes and macrophages produce large amounts of $O_2^-$ and $H_2O_2$ (1). Oxygen free radicals destroy lipids by a process called lipid peroxidation. In synovial fluid (SF) from RA patients, degradation products of lipid peroxidation can be detected (2, 3). In RA patients and in experimentally-induced arthritis in animals, superoxide dismutase (SOD), an enzyme destroying $O_2^-$, given systemically or locally, induces a decrease of inflammation (4, 5). In RA hyaluronic acid is depolymerized, although no hyaluronidase is present in SF and the proteases in SF are unable to degrade hyaluronic acid. Oxygen free radicals have been shown to cause depolymerization of hyaluronic acid (6, 7).

The deleterious effect of oxygen free radicals increases when iron is present. Although there is much debate about the exact mechanism, it is generally accepted that iron stimulates the formation of $OH^{\cdot}$, possibly by the reaction: $O_2^{-} + H_2O_2 \xrightarrow{Fe^{3+}} OH^{\cdot} + OH^{-} + O_2$. In most systems which have been investigated, this catalytic function of iron is blocked when it is bound to its specific binding proteins.

The large number of phagocytosing granulocytes present in RA SF will produce a considerable amount of oxygen free radicals and $H_2O_2$. The aim of this study was to investigate the protection in SF against oxygen free radicals and $H_2O_2$. The following proteins, known to protect in vitro, were measured in SF: 1) SOD, which catalyses the reaction $2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$; 2) catalase and glutathione peroxidase, which enzymatically reduce $H_2O_2$ to $H_2O$; 3) ceruloplasmin, which scavenges $O_2^{-}$; 4) transferrin and ferritin, which are iron binding proteins.

Lipid peroxidation of rat liver microsomes induced by oxygen free radicals was used as a model. The effect of oxygen free radicals in this system can be inhibited by serum and SF. Factors responsible for the inhibition were investigated.

## MATERIALS AND METHODS

### Synovial fluid and sera

SF was obtained from 7 patients with traumatic knee lesions, 6 patients with osteoarthritis (OA) of the knee and 17 patients with classic of definite RA as defined by the American Rheumatism Association criteria (8). Sera were also obtained from the last 2 groups. Heparin was added to the SF samples. They were then centrifuged for 10 minutes at 3,000 revolutions per minute and the supernatants were stored at -70°C. Samples containing more than 15 mg hemoglobulin per l were excluded.

### Superoxide dismutase

SOD activity was determined using the cytochrome c reduction inhibition method of McCord (9). The SOD concentration was expressed in mg using bovine erythrocyte SOD (Boehringer, Mannheim, FRG) as a standard (lot 1071101, 5,000 units/mg). Under the applied conditions,

SF produced no spontaneous cytochrome c reduction. Ceruloplasmin at the concentration present in SF had no significant effect on the assay. SOD added to SF was determined to have a recovery of 89%, so no significant inhibition was present in SF.

Catalase

Catalase was measured by tracing the degradation of $H_2O_2$ spectrophotometrically, according to the method of Aebi (10). The catalase activity in SF could be blocked effectively by 10 μM $NaN_3$. Catalase added to SF was determined to have a recovery of 90%.

Glutathione peroxidase

Glutathione peroxidase content was determined according to the method of Paglia and Valentine (11). One unit corresponds to 1 μmole NADPH oxidation per minute. The recovery of the enzyme in SF was 100%. Ceruloplasmin and transferrin levels were estimated by single radial immunodiffusion (12). Ferritin content was determined using an enzyme immunoassay (Ferrizyme, Abbott Laboratories, Chicago, IL).

Measurement of inhibition of $O_2^-$-induced lipid peroxidation

Lipid peroxidation of rat liver microsomes isolated from Wistar rats (13) was performed as follows: the incubation mixture contained (in final concentrations) 33 mM Tris-HCl (pH 6.8), 0.33 mM xanthine, 0.133 mg/ml dialysed xanthine oxidase, 0.12 mM $FeCl_3$, 2 mM ADP, and 1 mg protein/ml microsomes. The final volume was 3 ml. The incubation was performed at 37°C and started by the addition of xanthine oxidase. At indicated times, samples were drawn and thiobarbituric acid-reacting products were measured (14).

Serum or SF was applied to a 100 x 1,6 cm column of Sephadex G-200 and eluted at a flow rate of 6.5 ml/hour with 50 mM Tris-HCl (pH 7.4) with 0.1 M NaCl. Fractions of the eluate were assayed for the capacity to inhibit lipid peroxidation of rat liver microsomes and for the concentration of transferrin and ceruloplasmin.

Serum and SF free from ceruloplasmin were prepared with a column of human ceruloplasmin antibody linked to CNBr-activated Sepharose 4B. Before chromatography SF was pretreated with hyaluronidase to decrease the viscosity. The eluate was dialyzed against 2 mM sodium phosphate

(pH 7.0) and concentrated to the original protein concentration. The final preparations were checked for the presence of ceruloplasmin. Human ceruloplasmin type X was from Sigma Chemical Co., St. Louis, MO.

## Statistical analysis

Significance of differences in concentrations was determined by Student's t-test.

## RESULTS

### TOTAL PROTEIN

SF from patients with traumatic knee lesions contained 42 ± 8 mg protein/ml (mean ± SD), SF from patients with OA contained 49 ± 13 mg protein/ml, and RA SF contained 56 ± 7 mg protein/ml.

### SUPEROXIDE DISMUTASE, CATALASE, AND GLUTATHIONE PEROXIDASE

SOD activities were found to be as follows: 1.2 ± 0.6 mg/l in SF from patients with traumatic knee lesions, 1.3 ± 0.3 mg/l in OA SF, and 1.4 ± 0.6 mg/l in RA SF. No significant differences were found between these groups (Figure 1).

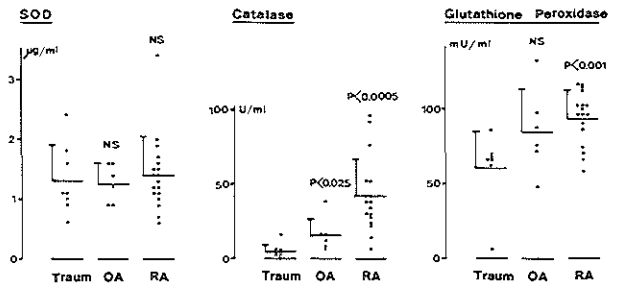



Fig.1: Superoxide dismutase (SOD), catalase, and glutathione peroxidase activities in synovial fluid (mean±SD) of patients with traumatic knee lesions (Traum), osteoarthritis (OA), and rheumatoid arthritis (RA). P values for OA and RA versus Traum are shown; NS= not significant.

The catalase activity in SF from patients with traumatic knee lesion was low: 3 ± 2 units/ml. In OA SF it was significantly increased to 16 ± 11 units/ml ($P < 0.025$) and in RA SF the highest activity was found: 42 ± 25 units/ml (Figure 1). The concentration in RA patients was significantly higher than in OA ($P < 0.025$) and trauma patients ($P < 0.0005$). Four of the RA patients had received aurothioglucose; they had very high catalase levels: 78 ± 20 units/ml. The increase of catalase in OA and RA cannot be explained by lysis of small amounts of erythrocytes. The accepted maximal amount of hemoglobin for used SF was 15 mg/l. Lysis of erythrocytes resulting in 15 mg/l hemoglobin in SF increased catalase activity by only 3 units/ml.

Glutathione peroxidase in SF from patients with traumatic knee lesions was 60 ± 25 mU/ml, in OA SF it was 85 ± 29 mU/ml, and in RA SF the activity increased significantly to 93 ± 18 mU/ml ($P < 0.001$ versus trauma patients) (Figure 1).

## TRANSFERRIN, FERRITIN AND CERULOPLASMIN

The concentrations of transferrin, ferritin and ceruloplasmin in SF from the 3 groups are shown in Figure 2. Transferrin was not significantly elevated in OA SF or in RA SF. The ferritin concentration in RA SF was strongly increased. In RA SF the average ceruloplasmin concentration was twice as high as in SF from trauma patients, and in OA SF the amount was also elevated.

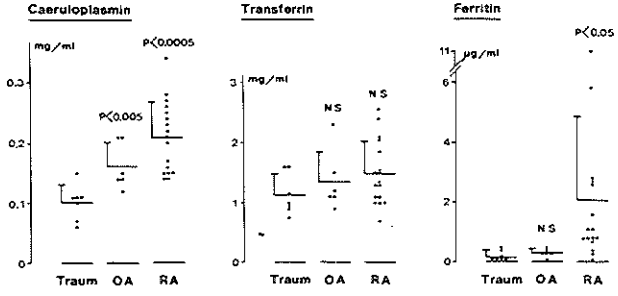



Fig.2: Ceruloplasmin, transferrin, and ferritin concentrations in synovial fluid in patients with traumatic knee lesions (Traum), osteoarthritis (OA) and rheumatoid arthritis (RA). P values for OA and RA versus Traum are shown; NS= not significant.

The distribution of these 3 proteins between serum and SF from the patients is shown in Table 1. Both transferrin and ceruloplasmin were present in SF in a concentration below the serum concentration. The SF/serum concentration ratio was slightly, but not significantly, higher in RA than in OA. Ferritin was present in SF from RA patients in a concentration 15 times higher than in the corresponding serum.

Table 1. Distribution of ceruloplasmin, transferrin and ferritin between serum and synovial fluid of patients with osteoarthritis and rheumatoid arthritis*.

| | SF | Serum | SF/serum ratio | P |
|---|---|---|---|---|
| Ceruloplasmin, mg/ml | | | | |
| OA (n= 6) | 0.162±0.039 | 0.350±0.063 | 0.46± 0.09 | NS |
| RA (n=17) | 0.209±0.059 | 0.423±0.148 | 0.50± 0.14 | (OA vs RA) |
| Transferrin, mg/ml | | | | |
| OA (n= 6) | 1.35 ±0.51 | 2.10 ±0.81 | 0.64± 0.08 | NS |
| RA (n=17) | 1.49 ±0.53 | 2.18 ±0.77 | 0.71± 0.22 | (OA vs RA) |
| Ferritin, g/ml | | | | |
| RA (n=17) | 2.09 ±2.77 | 0.15 ±0.24 | 14.8 ±13.6 | |

* Values are mean ± SD. SF = synovial fluid; OA = osteoarthritis; RA = rheumatoid arthritis; NS = not significant.

INHIBITION BY SERUM AND SF OF $O_2^-$-INDUCED LIPID PEROXIDATION

Peroxidation of rat liver microsomes, induced by $O_2^-$ generated by xanthine and xanthine oxidase in the presence of $Fe^{3+}$, could be blocked by addition of serum and SF from both RA patients and controls. The inhibition was concentration-dependent, and about 3% serum or about 6% SF resulted in complete inhibition. To determine which fraction was responsible for this inhibition, G-200 gel filtration was performed. In the eluate the capacity to inhibit the lipid peroxidation was measured. The G-200 gel filtration pattern of RA SF (Figure

3) is representative for the SF and sera both of RA patients and controls.

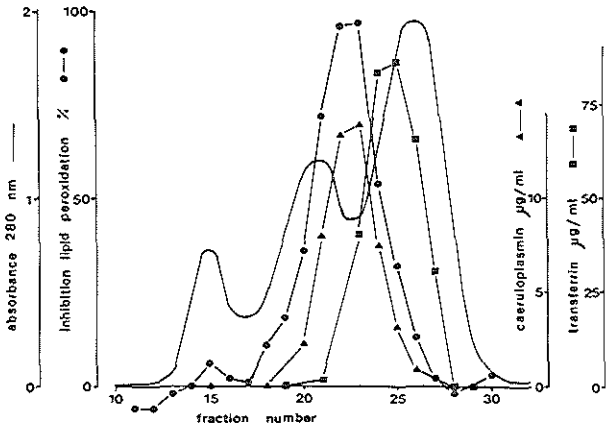



Fig.3: Sephadex G-200 gel filtration of synovial fluid from a patient with rheumatoid arthritis.

For all samples, maximal inhibition of the lipid peroxidation was found between the second and third peaks of absorption at 280 nm. Occasionally a small peak at the void volume was found; this was not further investigated. Ceruloplasmin and transferrin levels were determined in the fractions obtained. The ceruloplasmin concentration and the inhibition of the lipid peroxidation in individual fractions conformed closely to the same pattern. From the elution pattern it can be concluded that under these conditions, transferrin offers almost no protection against oxygen free radicals.

To investigate the possibility that ceruloplasmin was able to inhibit lipid peroxidation in the described system, the inhibition of purified human ceruloplasmin was measured. Ceruloplasmin was able to inhibit peroxidation of rat liver microsomes in a concentration-dependent manner. The necessary concentration was of the same order as found in serum and SF.

Serum and SF from control and RA patients were treated with human anti-ceruloplasmin to obtain fluids free of ceruloplasmin. The capacity of this serum and SF to inhibit lipid peroxidation was compared

with that of the original serum or SF. Figure 4 shows the effect of removing ceruloplasmin on the inhibition of lipid peroxidation of rat liver microsomes. In all groups the capacity to inhibit lipid peroxidation was decreased by 70% with removal of ceruloplasmin.

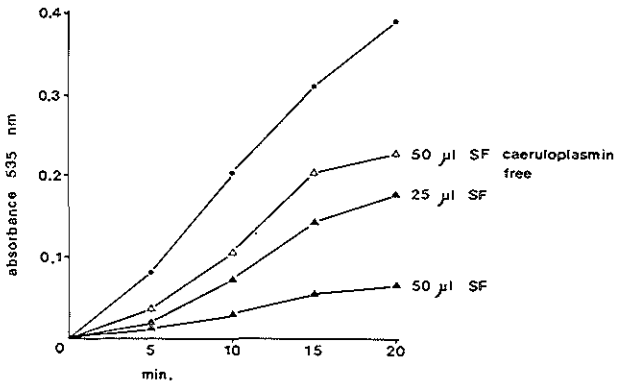



Fig. 4: Superoxide-induced lipid peroxidation of rat liver microsomes, followed by measurement of thiobarbituric acid -reacting products. Complete system ( ●—● ); final volume 3 ml. Effect of synovial fluid (SF) from a patient with rheumatoid arthritis ( ▲—▲ ), and the same fluid after removal of ceruloplasmin ( △—△ ).

**DISCUSSION**

There is much evidence to suggest that oxygen free radicals and $H_2O_2$ are closely involved in the pathogenesis of RA. Granulocytes are strongly increased in number in RA SF and produce large amounts of $O_2^-$ and $H_2O_2$ during the phagocytosis of immune complexes and other materials (1). Oxygen free radicals and other secondarily formed radicals are possibly responsible for at least part of the joint destruction. Several substances are known to protect against oxygen free radicals in vitro, but only a small number of investigations have been performed on detecting these protectors in RA.

Conflicting data are presented in the literature about the concentraton of superoxide dismutase in RA SF. Blake et al. found no SOD at all in RA SF (15). Igari et al. (16), however, showed a low concentration in OA SF but a 4 times higher level in RA SF. The concentration of SOD in granulocytes was decreased by 40% in juvenile RA patients (17).

Banford et al. (18) measured SOD in erythrocytes in patients with

RA. The SOD concentration of hemolysate was decreased in RA; however, a decrease in hemoglobin concentration was also found. The low concentration of SOD in hemolysate found in that study can be explained by the anemia commonly found in RA.

In the present study a low SOD concentration in SF from patients with traumatic knee lesions and with OA was found, in agreement with the results reported by Igari et al. for OA. In contrast with Igari's findings, the SOD concentration in RA was not elevated in our study. It is very doubtful that this low SOD concentration can protect against oxygen free radicals. In various cell types and tissues, SOD activity is 18-440 times higher, and in in vitro systems higher concentrations are needed to protect against oxygen free radicals.

Catalase and glutathione peroxidase are capable of detoxifying $H_2O_2$. Blake et al. (15) found low concentrations of catalase in RA SF, but no reference group was investigated. We estimated almost no catalase activity in SF from trauma patients. In OA SF the concentration was elevated, and in RA SF a further increase occurred. Within the RA group 4 patients who received aurothioglucose had very high catalase levels. Although catalase is significantly increased in RA, we agree with Blake et al. that the concentration is too low to expect considerable protection against $H_2O_2$ in SF.

Glutathione peroxidase was elevated in RA SF compared with SF from trauma patients. This increase was nearly as great as the increase of total protein found in RA SF. No increase in transferrin concentration was found in RA SF. The concentration is, however, high enough to bind about 4 times the amount of iron known to be present in SF (19). In most systems transferrin-bound iron is not able to catalyze the formation of $OH^{\cdot}$. Thus, before iron can have a deleterious effect, it must be separated from transferrin. Another possibility is that iron is bound very tightly in a small complex which prevents binding to transferrin.

Ferritin was strongly increased in RA SF compared with both OA and traumatic SF. This is in agreement with the results of Blake et al. (20). The ferritin concentration in RA SF was on the average 15 times higher than in the corresponding serum. It is unlikely that ferritin in RA SF is derived from serum, which is the case for most other serum proteins, such as ceruloplasmin and transferrin. The

concentration of these proteins in SF is less than the corresponding serum value. We favor the hypothesis that ferritin is synthesized by synovial membrane cells and enters SF by secretion of by release from dying cells. The high production of ferritin by synovial cells, as shown by Muirden et al. (21), is a protective reaction to the continuous iron release in SF, due to blood loss and the resulting breakdown of hemoglobin. Ferritin and transferrin can both play a dual role: 1) protection against oxygen free radicals by binding free iron, or 2) stimulation of $OH^{\cdot}$ production by release of free iron from the binding protein. In this context the percentage of iron saturation could be very important.

Ceruloplasmin has been shown to possess $O_2^-$ scavenging activity (22). The present investigations confirm that this acute phase reactant is elevated in RA SF (23, 24).

There are several potential protectors against oxygen free radicals in SF, but the relative importance of these proteins is unknown. Stocks et al. (25) have investigated inhibition of lipid peroxidation by serum. They used brain homogenate as a lipid peroxidating system, which could be blocked by small amounts of serum. They concluded that the protection by serum against oxygen free radical induced lipid peroxidation was based on the presence of transferrin and another protein, most likely ceruloplasmin. In the present study $O_2^-$ induced lipid peroxidation of rat liver microsomes was used. Serum and SF from both controls and RA patients blocked this process.

Gel filtration of serum or SF from both groups showed a peak at which lipid peroxidation was inhibited. The position of this peak did not correspond with transferrin levels, but did correspond with ceruloplasmin levels. The postulated role of ceruloplasmin was strengthened by the fact that purified human ceruloplasmin was able to inhibit lipid peroxidation in the system used on the same order of concentrations as were present in serum or SF. Furthermore, the inhibition of lipid peroxidation by ceruloplasmin-free serum or SF was diminished by about 70% compared with the corresponding complete serum or SF.

Ceruloplasmin is definitely shown to be an important protector against oxygen free radicals in serum and synovial fluid both in controls and rheumatoid arthritis patients. Our system is not appropriate for detecting a possible inhibitory effect of transferrin or

other iron binding proteins, since rather high concentrations of iron were added.

ACKNOWLEDGEMENTS

We wish to thank Dr. H.G. van Eijk for discussions about iron metabolism, Mr M.J. Kroos for his help in the ferritin determinations. Dr. T.A. Out and Mrs. A. van Rooijen from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service for the determinations of ceruloplasmin and transferrin. Dr. J.R.W. ten Kate for providing specimens of synovial fluid from patients with traumatic knee lesions, and Ms. A.C. Hanson for typing the manuscript.

REFERENCES

1. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanism: the production by leucocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741, 1973.
2. Lunec J, Halloran SP, Whithe AG, Dormandy TL: Free-radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J Rheumatol 8:233, 1981.
3. Muus P, Bonta IL, Den Oudsten SA: Plasma levels of malondialdehyde, a product of cyclo-oxygenase-dependent and independent lipid peroxidation in rheumaotid arthritis: a correlation with disease activity. Prostaglandins Med 2:63, 1979.
4. Goebel KM, Storck U, Neurath F: Intrasynovial orgotein therapy in rheumatoid arthritis. Lancet i:1015, 1981.
5. Huber W, Menander-Huber KB, Saifer MGP, Dang PHC: Studies on the clinical and laboratory pharmacology of drug formulations of bovine Cu-Zn superoxide dismutase (orgotein) In: Perspectives in Inflammation, Willoughby DA, Giroud JP and Velo GP (eds) MTP Press, 527, 1977.
6. McCord JM: Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science 185:529, 1974.
7. Greenwald RA, Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum 23:455, 1980.

8. Ropes MW, Bennett GA, Cobb S, Jacox R, Jessar RA: 1958 revision of diagnostic criteria for rheumatoid arthritis. Bull Rheum Dis 9:175, 1958.
9. McCord JM, Crapo JD, Fridovich I: Superoxide dismutase assays: a review of methodology, Superoxide and Superoxide Dismutases. Michelson AM, McCord JM, Fridovich I (eds) Academic Press, London, 11, 1977.
10. Aebi H: Catalase, Methods of Enzymatic Analysis. Second edition, Bergmeyer HU (ed) Academic Press, New York, 673, 1974.
11. Paglia DE, Valentine WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158, 1967.
12. Mancini G, Carbonara AO, Heremans JF: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235, 1965.
13. Wills ED: Lipid peroxide formation in microsomes: general consideration. Biochem J 113:315, 1969.
14. Ottolenghi A: Interaction of ascorbic acid and mitochondrial lipides. Arch Biochem Biophys 79:355, 1959.
15. Blake DR, Hall ND, Terby DA, Halliwell B, Gutteridge JMC: Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid arthritis. Clin Sci 61:483, 1981.
16. Igari T, Kaneda H, Horiuchi S, Ono S: A remarkable increase of superoxide dismutase activity in synovial fluid of patients with rheumatoid arthritis. Clin Orthop 162:282, 1982.
17. Rister M, Bauermeister K, Gravert U, Gladtke E: Superoxide dismutase and glutathione peroxidase in polymorphonuclear leucocytes. Eur J Pediatr 130:127, 1979.
18. Banford JC, Brown DH, Hazelton RA, McNeil CJ, Sturrock RD, Smith WE: Serum copper and erythrocyte superoxide dismutase in rheumatoid arthritis. Ann Rheum Dis 41:458, 1982.
19. Niedermeier W, Griggs JH: Trace metal composition of synovial fluid and blood serum of patients with rheumatoid arthritis. J Chronic Dis 23:527, 1971.
20. Blake DR, Bacon PA, Eastham EJ, Brigham K: Synovial fluid ferritin in rheumatoid arthritis. Brit Med J 281:715, 1980.

21. Muirden KD, Fraser JRE, Clarris B: Ferritin formation by synovial cells exposed to haemoglobin in vitro. Ann Rheum Dis 26:251, 1967.
22. Goldstein IM, Kaplan HB, Edelson HS, Weissmann G: Ceruloplasmin: a scavenger of superoxide anion radicals. J Biol Chem 254:4040, 1979.
23. Niedermeier W: Concentration and chemical state of copper in synovial fluid and blood serum of patients with rheumatoid arthritis. Ann Rheum Dis 24:544, 1965.
24. Scudder PR, McMurray W, White AG, Dormandy TL: Synovial fluid copper and related variables in rheumatoid and degenerative arthritis. Ann Rheum Dis 37:71, 1978.
25. Stocks J, Gutteridge JMC, Sharp RJ, Dormandy TL: The inhibition of lipid autoxidation by human serum and its relation to serum proteins and α-tocopherol. Clin Sci Mol Med 47:223, 1974.

CHAPTER 3.

# INTRACELLULAR AND EXTRACELLULAR SULPHYDRYL LEVELS IN RHEUMATOID ARTHRITIS

J.F. Koster, P. Biemond and A.J.G. Swaak

Ann Rheum Dis 45:44, 1986

SUMMARY

We detected no difference in the reduced glutathione content of erythrocytes obtained from patients with rheumatoid arthritis (RA) and controls. The stability of glutatione to oxidative stress (cumene hydroperoxide) was also the same. Although measured in the erythrocyte, our results indicate that changes in intracellular reduced glutathione are not involved in the aetiology of RA. Serum from patient with RA had a significantly reduced ($P < 0.01$) sulfhydryl (SH) concentration (415 $\pm$ 89 (SD) µmol/l) compared with controls (583 $\pm$ 74 µmol/l). This was also valid if the SH groups were expressed per gram of protein. Serum and synovial fluid from RA patients contained similar levels of SH groups (µmol/g protein).

Key words: glutathione, erythrocyte membrane, oxygen free radicals, oxidative stress, cumene hydroperoxide, antioxidants.

## INTRODUCTION

It has been reported that the number of free SH groups in the sera of rheumatoid arthritis (RA) patients is depressed (1, 2). This depression is associated with the activity of the disease (2). Hall et al. (3) have shown *in vitro* that $H_2O_2$ formed during phagocytosis by granulocytes diminishes the amount of free SH groups. These authors propose that serum SH groups act as important extracellular scavengers of peroxides and are therefore helpful in protecting the surrounding tissues. However, it is unlikely that $H_2O_2$ is formed in the intravascular compartment, whereas $H_2O_2$ would be expected in the synovial fluid of RA patients due to the activity of phagocytosing cells. It is possible therefore that the concentration of sulfhydryl groups/g protein might be lower in synovial fluid than in serum. In this report we compared the concentration of SH groups in serum and synovial fluid of RA patients.

An important, non-protein, free SH is glutathione (GSH). This tripeptide is located almost entirely intracellularly and the negligible amount present in serum can be ignored for the purposes of this study. GSH is very important for normal cell functions. In the context

of this report GSH has an important role in the defence against oxidative stress. For example, glutathione peroxidase is involved in the destruction of free peroxidated fatty acids and $H_2O_2$ (4). Furthermore, glutathione is the cosubstrate for the peroxidation inhibiting protein which is capable of destroying hydroperoxide fatty acids located in phospholipids (5). Glutathione peroxidase does not possess this latter activity (4). Oxidised glutathione (GSSG) is reduced by the NADPH (reduced nicotinamide adenine dinucleotide phosphate) dependent enzyme glutathione reductase. NADPH, formed by the pentose phosphate shunt, is important, as it is not only the total amount of GSH which is important for the cell but also the capacity of the cell to reduce the formed GSSG.

It has been reported (6) that the amount of GSH in erythrocytes of RA patients increases after treatment with penicillamine.
It has also been claimed (7) that GSH decreases during relapses and increases during remissions. Since the amount of GSH of RA patients and controls does not differ very much, we have investigated the capacity of erythrocytes from RA patients to reduce GSSG.

## MATERIALS AND METHODS

Serum, synovial fluid, and erythrocytes were obtained from patients with classic or definite RA as defined by the American Rheumatism Association criteria (8). Controls and patients were age matched and all below the age of 55.

Protein determinations were performed according to Lowry et al. (9). SH groups were assayed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as follows: 50 µl serum, 750 µl 0.1 M phosphate buffer (pH 7.4) and 200 µl DTNB (2 mM) were incubated at 37°C for 5 min. The absorbance at 412 nm was measured and a molar extinction coefficient of 13,600 was used. GSH was determined according to Beutler et al. (10). The capacity of cells to reduce oxidised glutathione (the so called glutathione stability) was determined according to Koster et al. (11).

## RESULTS

### SERUM AND SYNOVIAL FLUID SH GROUPS

Table 1 shows the amount of SH groups present in the sterum and synovial fluid of RA patients and in the serum of controls. The absolute concentration of SH in serum from RA patients was significantly lower than that of healthy controls ($P < 0.01$), when expressed as either µmol/l or µmol/g protein. Synovial fluid samples were obtained from the same RA patients. The synovial fluid SH concentration was lower than in serum. No difference was found when the SH concentration was expressed as µmol/g protein.

Table 1. The level of SH groups in the serum and synovial fluid of RA patients (n=5) and in the serum of controls (n=5).

| | SH (µmol/l) | Protein (mg/ml) | SH/g protein (µmol/g protein) |
|---|---|---|---|
| Control serum | 583±74 | 70.6±2.2 | 8.26±1.09 |
| RA patient serum | 415±89* | 70.8±6.7 | 5.85±1.06* |
| RA patient synovial fluid | 257±64 | 41.4±6.9 | 6.23±1.24@ |

The values are mean ± SD.
* $P < 0.01$ compared with control.
@ Not significant compared with RA serum.

### GLUTATHIONE STABILITY IN ERYTHROCYTES FROM RA PATIENTS AND HEALTHY CONTROLS

There was no difference in the amount of GSH (nmol/ml(µmol/l)packed cells) in erythrocytes from RA patients and controls (Table 2).

Table 2. The GSH level in erythrocytes from RA patients and controls.

| | Control (n=4) | RA (n=6) |
|---|---|---|
| GSH (nmol/ml packed cells)* | 1772±144 | 1898±85 |

Values are mean ± SD.
* nmol/ml=μmol/l.

Fig. 1. compares the ability of erythrocytes obtained from RA patients and healthy controls to regenerate GSH after peroxidative stress. There is the same rapid depletion of GHS upon addition of cumene hydroperoxide and a similar rate of GSH restoration. This indicates that the capacity to maintain adequate GSH levels under peroxidative circumstances is the same for both groups.

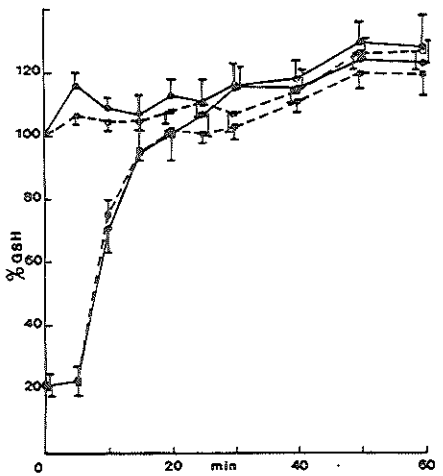



Fig. 1: The reduced glutathione content of intact erythrocytes from RA patients (n=6) and controls (n=4) during incubation with cumene hydroperoxide (0.5 mM) in the presence of glucose (5 mM). Cumene hydroperoxide was added at zero time, and the values are expressed as a percentage of the initial glutathione content. Controls o—o and •—• ; RA patients □—□ and ■—■ Open symbols are in the absence and closed symbols in the presence of cumene hydroperoxide.

## DISCUSSION

We have confirmed that sera from RA patients have a reduced amount of SH groups compared with controls. The reduction in serum SH groups is not simply a result of the decrease in serum albumin as shown by Thomas and Evans (12). Hall et al. (3) suggested that the diminished amount of SH groups may be due to oxidation by $H_2O_2$ generated by stimulated phagocytes. We were able to confirm these data (unpublished results). In RA patients without extra-articular lesions it is unlikely that circulating granulocytes are stimulated and so would not produce $H_2O_2$. In the synovial fluid $H_2O_2$ is to be expected due to phagocytosis. It therefore seemed reasonable to assume that the amount of SH (µmol/g protein) would be lower in the synovial fluid than in the sera of RA patients. However, our data showed that there was no difference in SH groups (per g protein) in these two compartments. It is possible that this is due to a rapid exchange of proteins between the synovial fluid and serum. Another possibility is that the synovial fluid contains a factor(s) which protect(s) the proteins effectively.

Treatment of RA patients with penicillamine leads to an increase in the amount of erythrocyte GSH (6, 7). Although the absolute amount of GSH is important, in order to maintain cellular functions and provide a barrier against peroxidative destruction, the capacity to reduce the formed GSSG is also very important. This reaction enables the cell to withstand stress longer than would otherwise be possible if GSH were not regenerated. We have shown that there is no difference in GSH content of erythrocytes from RA patients and controls. If alterations of GSH levels did have a role in the aetiology of RA, it is possible that the capacity to regenerate GSH during oxidative stress could be reduced. However, erythrocytes from controls and RA patients were equally effective in regenerating GSH when challenged with cumene hydroperoxide. It is therefore unlikely that the increase in erythrocyte GSH, observed on administration of penicillamine, can explain the disease remissions. Furthermore it is not known, at least to our knowledge, what happens to the GSH level in erythrocytes from controls treated with penicillamine.

ACKNOWLEDGEMENTS

The authors thank Ms R.G. Slee for the determination of reduced glutathione and Mr C.M. Beindorff for technical assistance, Dr. G. Elliot for correcting the English and Ms A.C. Hanson for preparing the manuscript.

REFERENCES

1. Lorber A, Pearson CM, Meredith WL, Gantz-Mandell LE: Serum sulfhydryl determinations and significance in connective tissue diseases. Ann Intern Med 61:423, 1964.
2. Haataja M: Evaluation of the activity of rheumatoid arthritis. A comparative study on clinical symptoms and laboratory tests with special reference to serum sulfhydryl groups. Scand J Rheumatol 4(suppl 7):3, 1975.
3. Hall ND, Maslen CL, Blake DR: The oxidation of serum sulfhydryl groups by hydrogen peroxide secreted by stimulated phagocytic cells in rheumatoid arthritis. Rheumatol Int. 4:35, 1984.
4. McCay PB, Gibson, DD, Hornbrook KR: Glutathione-dependent inhibition of lipid peroxidaton by a soluble, heat-labile factor, not glutathione peroxidase. Fed Proc. 40:199, 1981.
5. Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C: Purification from pig liver of protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity in phosphatidyl choline hydroperoxides. Biochim. Biophys. Acta 710:197, 1982.
6. Munthe E, Guldal G, Jellum E: Increased intracellular glutathione deriving penicillamine treatment for rheumatoid arthritis. Lancet ii:1126, 1979.
7. Munthe E, Käss E, Jellum E: D-Penicillamine-induced increase in intracellular glutathione correlating to clinical response in rheumatoid arthritis. J Rheumatol 8(suppl 7):14, 1981.
8. Ropes MW, Bennett GA, Cobb S, Jacox R, Jessar RA: 1958 revision of diagnostic criteria for rheumatoid arthritis. Bull Rheum Dis 9:175, 1958.

9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurements with the Folin phenol reagents. J Biol Chem 193:265, 1951.
10. Beutler E, Duron O, Kelly BM: Improved method for the determination of blood glutathione. J Lab Clin Med 61:882, 1963.
11. Koster JF, Slee RG, Rutten-Van Beysterveld CCM, Montfoort A: The effect of (13 OOH) linoleic acid on human erythrocytes and on erythrocyte ghosts. Biochim Biophys Acta 754:238, 1983.
12. Thomas J, Evans PH: Serum protein changes in coal workers' pneumoconiosis. Clin Chim Acta 60:237, 1975.

CHAPTER 4.

# IRON MOBILIZATION FROM FERRITIN BY SUPEROXIDE DERIVED FROM STIMULATED POLYMORPHONUCLEAR LEUKOCYTES. POSSIBLE MECHANISM IN INFLAMMATION DISEASES

P. Biemond, H.G. van Eijk, A.J.G. Swaak and J.F. Koster

J Clin Invest 73:1576, 1984

ABSTRACT

During inflammation, the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are produced by stimulated polymorphonuclear leukocytes and macrophages. The toxic effects of these reactive oxygen intermediates increase when traces of iron are present, because iron catalyzes the formation of the hydroxyl radical ($OH^\cdot$). Partially saturated iron-binding proteins, such as transferrin and ferritin, are unable to catalyze $OH^\cdot$ formation in vitro. Mobilization of iron from these proteins is necessary for iron stimulation of $OH^\cdot$ formation. This paper reports that stimulated polymorphonuclear leukocytes mobilize iron from human and horse ferritin, but not from human transferrin. Iron release from ferritin depends on $O_2^-$ because it can be prevented by the addition of superoxide dismutase. Catalase and dimethylsulfoxide have no inhibitory effect on iron mobilization. The efficiency of the iron release increases at low levels of $O_2^-$ production. Only $O_2^-$ produced by granulocytes is sufficient for iron mobilization, because solid potassium superoxide is also able to release iron from ferritin. We propose that this reaction may potentiate the formation of the $OH^\cdot$ radical in inflammatory states.

INTRODUCTION

During inflammation, stimulated polymorphonuclear leukocytes (PMN) and macrophages produce large amounts of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (1). These products can be toxic by themselves, but their detrimental effects are strongly increased in the presence of iron, able to catalyze the formation of the hydroxyl radical ($OH^\cdot$) by the following overall reaction: $O_2^- + H_2O_2 \xrightarrow{\text{iron}} O_2 + OH^- + OH^\cdot$. Nearly all the iron present in the body is located in haem-containing proteins and in iron-binding proteins such as transferrin, ferritin, lactoferrin, or in enzymes. In in vitro systems, protein-bound iron is often unable to catalyze $OH^\cdot$ formation (2, 3, 4). Iron-binding proteins are even able to inhibit destruction by oxygen free radicals by binding of iron (5, 6).

The present investigations are focussed on the mobilization of iron from ferritin or transferrin by $O_2^-$ produced during inflammation. Subsequently, mobilized iron could catalyze OH• formatin. Ferritin is very important in the storage of iron, but the exact physiological mechanism for iron mobilization from ferritin is still unknown. Reduction of $Fe^{3+}$ in the ferritin core to $Fe^{2+}$ seems to be essential (7). Superoxide can act as a reductant able to reduce $Fe^{3+}$ to $Fe^{2+}$. In this study we were able to show that $O_2^-$, derived from stimulated PMN, is able to mobilize iron from ferritin. The relevance of this finding in relation to rheumatoid arthritis (RA) is discussed.

## METHODS

### Reagents

Cadmium-free ferritin from horse spleen, 22% iron (50% saturated), catalase, cytochrome c and superoxide dismutase (SOD) from bovine erythrocytes were obtained from C.F. Boehringer & Sons, Mannheim, Federal Republic of Germany. Dimethylsulfoxide (DMSO) and 4,7 diphenyl-1,10-phenanthroline disulfonate acid sodium salt (bathophenanthroline) came from E. Merck, Darmstadt, Federal Republic of Germany. Human transferrin was from Behringwerke A.G., Marburg, Federal Republic of Germany. Potassium superoxide ($KO_2$) came from Fluka A.G., Buchs, Switzerland; and Sephadex G-50 medium was from Pharmacia Fine Chemicals, Uppsala, Sweden. 4β-Phorbol 12β-myrisate 13α-acetate (PMA) and horse spleen apoferritin were obtained from Sigma Chemical Co., St. Louis, MO. Sodium metrizoate-ficoll (Lymphoprep) came from Nyegaard & Co. Oslo, Norway.

### PMN leukocytes

PMN were isolated from defibrinated blood of healthy human volunteers by sodium metrizoate-ficoll centrifugation and by lysis of erythrocytes using a cold isotonic $NH_4Cl$ solution (8). The final cell suspension contained 95% PMN. At least 95% of the cells were viable, based on the exclusion of trypan blue. PMN were suspended in a solution containing: 137 mM NaCl, 5.4 mM KCl, 0.7 mM $NaH_2PO_4$, 0.8 mM

$MgSO_4$, 1.3 mM $CaCl_2$, 5.5 mM glucose, 25 mM Tris (pH 7.4 at 37°C) and 5 g/l of bovine serum albumin. This solution was also used as incubation buffer.

Ferritin iron mobilization by PMN

The incubation mixture contained in final concentrations: 0.5 - 1.0 x $10^6$ PMN/ml, 100 ng PMA/ml, 0.25 mg/ml horse spleen ferritin and 1 mM bathophenanthroline. The incubation at 37°C was started by adding PMN. At different times, samples were drawn and placed on ice. The samples were centrifuged for 5 minutes at 20,000 g and the absorption at 530 nm was measured with the incubation buffer as reference. The amount of iron release was calculated from the increase in the absorption. The extinction coefficient of bathophenanthroline is 22,140 $M^{-1}cm^{-1}$ (9). The same incubations were performed with 0.25 mg/ml human ferritin, which was isolated from human liver according to Penders et al. (10), and with 3 mg/ml human transferrin, which was 60% saturated with iron. All the incubations were carried out in duplo. Gel filtration of the iron-binding proteins on Sephadex G-50 were carried out to exclude interference by nonprotein-bound iron.

Efficiency of $O_2^-$-dependent iron mobilization

Using the same batch of PMN, both the $O_2^-$ production and the iron mobilization from horse spleen ferritin were measured at the same time. Iron release during 15 min was estimated as described above. $O_2^-$ production was measured with a mixture that contained 150 µM cytochrome c, 100 ng/ml PMA and 0.034-1.34 x $10^6$ PMN/ml, using the same incubation buffer. The incubation at 37°C was started by addition of PMN. After 15 min, the absorption at 550 nm was measured. The $O_2^-$ production was calculated from the increase of the absorption by using an extinction coefficient of 21,100 $M^{-1}cm^{-1}$ for cytochrome c. Control experiments showed that cytochrome c reduction by stimulated PMN could be blocked completely by 13 µg/ml SOD.

Ferritin iron mobilization by $KO_2$

0.25 mg/ml horse spleen ferritin and 1 mM bathophenanthroline were added to the above mentioned buffer without bovine serum albumin (pH 7.4). The absorption at 530 nm was measured, using the buffer as

reference. Solid $KO_2$ was added, and after the oxygen bubbles disappeared, the absorption was measured again. The addition of $KO_2$ was repeated several times. At the end of this experiment the pH was slightly increased to 8.0.

## RESULTS

Figure 1 shows that PMN, stimulated with PMA, are capable of mobilizing iron from horse spleen ferritin. The incubation mixture contained 0.25 mg/ml ferritin, which represented 980 uM iron. After 30 min, 0.5% of the iron was mobilized. If the incubation was performed at $0^oC$, or when no PMN were added, hardly any iron was mobilized (Table 1). There was also no significant iron release following incubation in the absence of ferritin or with iron-free horse ferritin (apoferritin). After stimulation, PMN produce large amounts of $O_2^-$ and $H_2O_2$. It is known that $O_2^-$ can act as a reducing agent; therefore, $O_2^-$ could be responsible for the iron mobilization from ferritin. Addition of SOD resulted in an inhibition of iron mobilization, which gives strong evidence that $O_2^-$ is involved. Addition of catalase showed a small increase in iron release, which can be explained by prevention of reoxidation of $Fe^{2+}$ by $H_2O_2$. DMSO, a scavenger of $OH^\cdot$ radicals, had no influence.

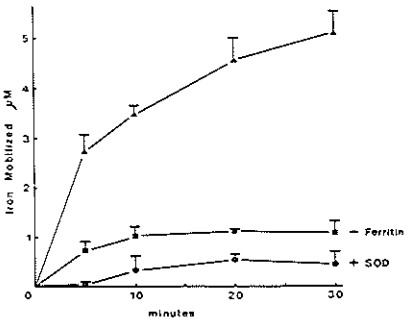



Fig.1: Iron mobilization from horse spleen ferritin by superoxide produced by stimulated PMN. The incubation mixture contained 0.25 mg/ml ferritin, $0.8 \times 10^6$ PMN/ml and 1mM bathophenanthroline to measure $Fe^{2+}$. Complete mixture (▲); 100 mg/ml SOD added (●); ferritin omitted (■). Mean and standard deviation of two incubations are shown.

Table 1. Iron mobilization from human and horse ferritin and transferrin by $O_2^-$ produced by stimulated PMN and the effect of SOD, catalase and DMSO

| | Iron mobilized in 30 min | Percentage of iron mobilization compared with the complete system without additions |
|---|---|---|
| | µM | % |
| Horse spleen ferritin (0.25 mg/ml, n=9) | 5.1±0.4 | 100 |
| Incubation at 0°C | | 0± 3 |
| Without ferritin | | 19± 3 |
| Apoferritin substituted for ferritin | | 16± 5 |
| Without PMN | | 10± 6 |
| Addition SOD (8.3 µg/ml) | | 45± 2 |
| Addition SOD (33.3 µg/ml) | | 25±10 |
| Addition catalase (1040 U/ml) | | 120± 9 |
| Addition DMSO (10 mM) | | 102± 7 |
| Human liver ferritin (0.25 mg/ml) | 3.2±0.4 | 100 |
| Addition SOD (33.3 µg/ml) | | 46± 4 |
| Human transferrin (3 mg/ml) Iron saturation 60% | 0.1±0.03 | |

All incubations were carried out with three different PMN preparations: the mean ± SD is shown. For each PMN preparation the incubations were performed in duplicate.

Similar results were obtained when human liver ferritin was added instead of horse spleen ferritin. The incubation mixture contained 980 μM iron bound in ferritin. After 30 min, 0.3% of the iron was mobilized. Iron mobilization from human ferritin was also inhibited by the addition of SOD. No measurable amount of iron could be mobilized from human transferrin. The total amount of iron present in the incubation mixture was considerably lower in cases of transferrin compared with cases of ferritin. Also, by increasing the transferrin concentration five times, practically no iron could be mobilized. A further increase in concentration of transferrin was technically impossible. Sephadex G-50 gel filtrations of the iron-binding proteins were performed before the incubations to exclude interference by nonprotein-bound iron.

Theoretically, one mole of iron can be mobilized by one mole of $O_2^-$. Therefore, the ratio of mobilized iron over total $O_2^-$ produced can be considered as a parameter for the efficiency by which $O_2^-$ is used for iron mobilization. This was tested by using varying amounts of PMN. Simultaneous measurements of $O_2^-$ production and iron release showed that the efficiency declined at higher $O_2^-$ production (figure 2).

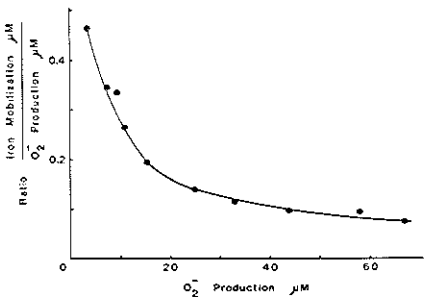



Fig. 2: Efficiency of iron mobilization from horse spleen ferritin by $O_2^-$ produced by PMN. Simultaneous $O_2^-$ production and iron release from ferritin by stimulated PMN were measured. $O_2^-$ production was varied by using different amounts of PMN (0.034-1.34 x$10^6$ ml). Incubation time was fixed at 15 min.

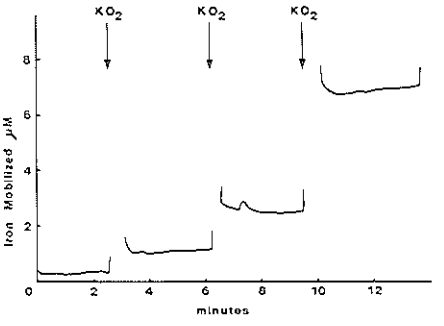



Fig. 3: Iron mobilization from horse spleen ferritin by $KO_2$. Assay mixture contained 0.25 mg/ml ferritin and 1mM bathophenanthroline. At indicated times some grains of $KO_2$ were added.

During stimulation, proteases are also released. These proteases possibly destroy ferritin, thereby liberating the iron that is subsequently reduced by $O_2^-$. Figure 3 shows, however, that addition of grains of solid $KO_2$ to ferritin caused mobilization of iron. This experiment showed that $O_2^-$ is capable of mobilizing iron from ferritin. $KO_2$-induced iron mobilization could not be inhibited by SOD. This can be explained by the observation that at very high levels of $O_2^-$, like in this experiment, spontaneous dismutation is much more important than SOD catalyzed dismutation (11).

## DISCUSSION

During inflammation, large amounts of $O_2^-$ and $H_2O_2$ are produced by stimulated PMN and macrophages (1). The destructive effects of these reactive oxygen intermediates are greatly enhanced by the presence of iron. This leads to the formation of the $OH^\cdot$ radical from $O_2^-$ and $H_2O_2$. *In vivo*, nearly all the iron is located in enzymes, haem-containing proteins, or in specific iron-binding proteins such as transferrin, ferritin, and lactoferrin.

Ferritin is the main protein in the storage of iron (12, 13). It consists of 24 polypeptide chains. There are two types of subunits (H and L) with molecular weights of 22,000 and 19,000, respectively. The polypeptide chains are arranged in a spherical protein shell with a central cavity. There are six channels through this shell. In one molecule of ferritin up to 4,500 iron atoms can be stored. Iron is present in ferritin largely as ferric oxyhydroxide, together with some phosphate. Deposition of iron in ferritin requires that iron be present in the ferrous state; molecular oxygen is also used in the process. The exact mechanism is still unrevealed. The mechanism of iron mobilization is also unclear. A reducing substance is needed to form ferrous iron. *In vivo* reduced riboflavin mononucleotides possibly play this role. Subsequently, the ferrous iron must be complexed with a suitable iron chelator.

Many investigations *in vitro* showed that protein-bound iron does not catalyze $OH^\cdot$ formation (2-4). Furthermore, prevention of $OH^\cdot$ production by lactoferrin and transferrin was found to be due to the binding of free iron present in the test system (5, 6). In contrast,

other studies showed that iron-binding proteins were able to stimulate $OH^{\cdot}$ formation (14-17). In these studies, however, the iron-binding proteins were fully saturated with iron, which seldom occurs *in vivo*, or no saturation was mentioned. So, most probably *in vivo*, the iron-binding proteins, which are partially saturated with iron, are incapable of catalyzing the production of the very aggressive $OH^{\cdot}$ radical. Gutteridge et al. (4) reported the presence of a small amount of nonprotein-bound iron in some extracellular fluids, for example synovial fluid. Until now, this interesting observation was not confirmed by others.

It is difficult to understand that "free iron" can exist in the presence of transferrin, only partially saturated with iron, because transferrin has a very high affinity for iron. The present study shows an alternative source of iron, which can catalyze $OH^{\cdot}$ formation. Stimulated PMN caused mobilization of iron from ferritin. The iron release could be blocked by SOD, but not by catalase or DMSO, indicating that $O_2^-$ was necessary in contrast to $H_2O_2$ and $OH^{\cdot}$. Catalase showed a small but reproducible increase in iron mobilization. This can be explained as follows: catalase inhibits oxidation of small amounts of free $Fe^{2+}$ to $Fe^{3+}$ by $H_2O_2$. $Fe^{3+}$ is unable to bind to bathophenanthroline, a known chelator, to estimate $Fe^{2+}$.

The efficiency of iron mobilization by $O_2^-$ was dependent on the amount of $O_2^-$ produced. At a low level of $O_2^-$ production per time unit a relatively greater amount of iron was mobilized than at a higher level of $O_2^-$ production per time unit. Stimulated PMN also caused release of iron from human liver ferritin. Again the iron release was $O_2^-$ dependent, for it could be inhibited by SOD. $O_2^-$ was not able to release measurable amounts of iron from human transferrin.

Stimulated PMN not only produce $O_2^-$ and $H_2O_2$, but also produce proteolytic enzymes. It is possible that the proteolytic enzymes degrade the protein part of ferritin, and that $O_2^-$ subsequently reduces $Fe^{3+}$ to $Fe^{2+}$, which can bind to bathophenanthroline. Although it cannot be excluded that proteolytic enzymes play an additional role in the measured iron mobilization, it is shown that $O_2^-$ on its own is able to release iron from ferritin. Addition of solid $KO_2$ to horse spleen ferritin resulted in a release of iron.

Iron release from ferritin is shown to be possible with xanthine

and xanthine oxidase, a well known source of $O_2^-$ (18, 19). It is doubtful whether xanthine and xanthine oxidase mobilize iron from ferritin by formation of $O_2^-$, because mobilization occurs much better when oxygen is absent and, in our knowledge, no literature data show that the release can be blocked by SOD. In preliminary investigations we were also able to release iron from ferritin by xanthine and xanthine oxidase, but it could not be blocked by SOD. Electrons are probably carried from the enzyme substrate complex to ferritin using a different carrier than $O_2^-$.

The conclusion drawn is that stimulated PMN and macrophages will mobilize iron if ferritin is present by an $O_2^-$ dependent mechanism. This iron can be used immediately for stimulating $OH^\cdot$ formation from $O_2^-$ and $H_2O_2$, which are produced by the same stimulated cells, before it will bind to transferrin.

The following pathophysiological consequences can be expected from the mobilization of iron from ferritin by stimulated PMN. There is a continuous flow of leukocytes to synovial fluid, predominantly PMN, reaching levels up to $7 \times 10^7$/ml. The PMN phagocytose actively immune complexes and other material. These cell numbers are much higher than those used in the present in vitro study. Thus, a continuous production of considerable amounts of $O_2^-$ can be expected. Although the ferritin concentration in synovial fluid of RA patients is much lower than the concentration used in our system, the continuous $O_2^-$ production by the invading PMN possibly releases enough iron to catalyze the formation of $OH^\cdot$. It is worth mentioning that RA synovial fluid contains much higher concentrations of ferritin ($2.09 \pm 2.77$ µg/ml) than normal synovial fluid ($0.23 \pm 0.17$ µg/ml) (20, 21). In the synovial membrane of RA patients the number of macrophages are increased and a large amount of ferritin is present (22); thus, iron mobilization can be expected. Iron release from ferritin by $O_2^-$ can also be important in other diseases when inflammatory cells are stimulated in the presence of ferritin. In haemochromatosis such a situation possibly occurs.

ACKNOWLEDGEMENTS

We wish to thank Dr. R.N. Lemon for correction of the English language and Mrs E.M.W. van Zaal-de Korte for preparing the manuscript.

REFERENCES

1. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanism. The production by leukocytes of superoxide, a potent bactericidal agent. J Clin Invest 52:741, 1973.
2. Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. FEBS Lett 92:321, 1978.
3. Winterbourn CC: Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate and superoxide. Biochem J 198:125, 1981.
4. Gutteridge JMC, Rowley DA, Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of "free" iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199:263, 1981.
5. Stocks J, Gutteridge JMC, Sharp RJ, Dormandy TL: The inhibition of lipid autoxidation by human serum and its relation to serum proteins and α-tocopherol. Clin Sci Mol Med 47:223, 1974.
6. Gutteridge JMC, Paterson SK, Segal AW, Halliwell B: Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J 199:259, 1981.
7. Jones T, Spencer R, Walsh C: Mechanism and kinetics of iron release fom ferritin by dihydroflavins and dihydroflavin analogues. Biochemistry 17:4011, 1978.
8. Weening RS, Roos D, Loos JA: Oxygen consumption of phagocytizing cells in human leucocyte and granulocyte preparations: a comparative study. J Lab Clin Med 83:570, 1974.
9. Bothwell TH, Charlton RW, Cook JD, Finch CA: Iron metabolism in man. Blackwell Scientific Publications Ltd, Oxford, England, 353, 1979.
10. Penders TJ, De Rooij-Dijk HH, Leijnse B: Rapid isolation of ferritin by means of ultracentrifugation. Biochim Biophys Acta 168:588, 1968.

11. Brawn K, Fridovich I: Superoxide radical and superoxide dismutase: threat and defense. Acta Physiol Scand Suppl 492:9, 1980.
12. Crichton RR: Structure and function of ferritin. Angew Chem Int Ed Engl 12:57, 1973.
13. Harrison PM, Banyard SH, Hoare RJ, Russell SM, Treffry A: The structure and function of ferritin. In: Iron Metabolism. Ciba Foundation Symposium 51 (new series). Elsevier/Excerpta Medica/ North Holland, Amsterdam 19, 1977.
14. McCord JM, Day ED: Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett 86:139, 1978.
15. Ambruso, DR, Johnston RB: Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fraction and an enzymatic generating system. J Clin Invest 67:352, 1981.
16. Bannister JV, Bannister WH, Hill HAO, Thornalley PJ: Enhanced production of hydroxyl radicals by the xanthine-xanthine oxidase reaction in the presence of lactoferrin. Biochim Biophys Acta 715:116, 1982.
17. Motohashi N, Mori I: Superoxide-dependent formation of hydroxyl radical catalysed by transferrin. FEBS Lett 157:197, 1983.
18. Mazur A, Green S, Saha A, Carleton A: Mechanism of release of ferritin iron in vivo by xanthine oxidase. J Clin Invest 37:1809, 1958.
19. Duggan DE, Streeter KB: Inhibition of ferritin reduction by pyrazolo [3,4d] pyrimidines. Arch Biochem Biophys 156:66, 1973.
20. Blake DR, Bacon PA, Eastham EJ, Brigham K: Synovial fluid ferritin in rheumatoid arthritis. Brit Med J 281:715, 1980.
21. Biemond P, Swaak AJG, Koster JF: Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid. Arthritis Rheum 27:760, 1984.
22. Muirden KD: Ferritin in synovial cells in patients with rheumatoid arthritis. Ann Rheum Dis 25:387, 1966.

CHAPTER 5.

# INTRA-ARTICULAR FERRITIN-BOUND IRON IN RHEUMATOID ARTHRITIS
## A FACTOR THAT INCREASES OXYGEN FREE RADICAL-INDUCED TISSUE DESTRUCTION

P. Biemond, A.J.G. Swaak, H.G. van Eijk and J.F. Koster

Arthritis Rheum, in press

SUMMARY

Iron mobilized from ferritin is able to convert superoxide and hydrogen peroxide, which are produced in large amounts in rheumatoid arthritis (RA), to the extremely toxic hydroxyl radical. We have found that synovial fluid ferritin is increased significantly in RA patients compared with controls. The high synovial fluid: serum ferritin ratio is compatible with the hypothesis that synovial fluid ferritin is derived from the synovial membrane. We found no difference in ferritin concentrations in the synovial membranes of RA patients compared with controls. Quantitative data regarding the amount of iron bound to ferritin showed that the level was 2.9 times higher in RA synovial membrane than in that of controls. Moreover, RA synovial fluid contained considerable amouts of iron bound to ferritin. Calculation of the iron saturation of ferritin revealed that RA synovial membranes contained a mean of 2,210 moles of iron per mole of ferritin, which was significantly elevated compared with the mean value of 1,500 moles found in the synovial membranes of the controls. The decreased saturation of ferritin in RA synovial fluid, compared with that in the synovial membrane, could be due to an uncompensated release of iron from ferritin, that has been induced by superoxide that is produced by stimulated granulocytes. The results demonstrate that in the joints of RA patients, sufficient ferritin loaded with iron is available to stimulate oxygen free radical damage.

INTRODUCTION

It is well known that there is a relationship between iron concentration and the development of arthritis. In rheumatoid arthritis (RA) there is an increased concentration of iron in the synovial fluid and synovial membrane (1). Arthritis develops as a result of iron accumulation in the joint in hemochromatosis, which is characterized by a systemic overload of iron, and in hemophilia, in which synovial iron deposition results from recurrent hemorrhages. Recently, treatment of RA patients with intravenous administration of iron has been shown to result in a flare-up of the arthritis (2).

The importance of oxygen free radicals in the pathogenesis of RA

is becoming widely recognized (3-6). After stimulation, granulocytes and macrophages produce large amounts of superoxide ($O_2^-$) and hydrogen peroxide (7). These products can be toxic in themselves, but their toxicity increases greatly in the presence of iron and results in the production of the extremely reactive hydroxyl radical, as follows:

$$O_2^- + H_2O_2 \xrightarrow{\text{iron}} O_2 + OH^- + OH^\cdot$$

Hydroxyl radical formation can only be catalyzed by free iron (8). The main biologic function of iron-binding proteins is prevention of the hydroxyl radical formation that is stimulated by free iron. Iron bound to transferrin or lactoferrin, which are extracellular iron-binding proteins, is unable to stimulate hydroxyl formation (9). In contrast, ferritin can increase oxygen free radical damage (10).

Ferritin is the main protein involved in the intracellular storage of iron (11, 12). Apoferritin consists of 24 polypeptide chains. There are two types of subunits, H and L, with molecular weights of 22,000 and 19,000, respectively. The polypeptide chains are arranged in a spherical protein shell, with a central cavity. There are six channels through this shell. The molecular weight of apoferritin is approximately 450,000. One molecule of apoferritin can store as many as 4,500 iron atoms. Iron is present in ferritin largely in the form of ferric oxyhydroxide, together with some phosphate. The mechanisms by which iron is deposited in, and mobilized from, ferritin are not known. Our previous investigations have shown that the superoxide produced by granulocytes is able to release iron from feritin, which makes it available for stimulation of hydroxyl radical formation (13).

Ferritin has been found to be increased in RA synovial fluid (14). This discovery was followed by speculations about its role in the pathogenesis of RA (15). The probability of such was diminished by a preliminary report that the ferritin in RA synovial fluid does not contain iron (16). This situation prompted us to study the distribution of ferritin in the joint, together with its iron saturation.

## PATIENTS AND METHODS

### Synovial fluid, synovial membrane, and serum samples

Synovial fluids, synovial membranes, and sera were obtained from patients with classic or definite RA, according of the American Rheumatism Association diagnostic criteria (17), from patients with osteoarthritis (OA), and from patients with traumatic knee lesions, for which meniscectomy was performed.

Hyaluronidase and heparin were added to the synovial fluid samples. The samples were centrifuged and then stored at -70°C. Samples containing >15 mg/l hemoglobin were excluded from this study.

Synovial membrane biopsy specimens were mechanically homogenized in 10 volumes of 10 mM sodium phosphate, pH 8.0, for 60 seconds at 4°C. The homogenate was centrifuged at 15,000 g for 20 minutes. The supernatant was used after filtration.

### Measurement of ferritin

The ferritin concentrations in sera, synovial fluids, and synovial membrane homogenate supernatants were determined by the Specto-Ferritin immunoassay (Ramco Laboratories, Houston, TX). Samples with ferritin concentrations >400 µg/l were diluted prior to use.

### Measurement of ferritin-bound iron

Iron-free laboratory materials and solutions were used. Ferritin-bound iron was measured by a modification of the method of Zuyderhoudt et al. (18). Antibodies to human ferritin (Dakopatts, Glostrup, Denmark) were immobilized. The ferritin antibody (1 ml) was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer's instructions. The sample was diluted with equal amounts of 0.15 M NaCl, and 1 ml was mixed with 9 mg of Sepharose-anti-ferritin containing 34 µg of immunoglobulin. This was mixed overnight at room temperature. The samples were then centrifuged for 10 min at 250 g, and the supernatant was carefully removed. No ferritin was detected in the supernatant.

The pellet was removed, washed 3 times with saline, and 0.1 ml of 12.5 M HCl was added. After 15 min, 0.1 ml of 142 mM ascorbic acid (fresh) and 0.6 ml of 7.5 M sodium acetate were added (pH range 4-5).

Iron was detected after the addition of 0.2 ml of 10 mM Ferene-S (Sigma, St. Louis, MO) by using the extinction coefficient, at 595 nm, of 35,500 $M^{-1}cm^{-1}$. This method resulted in ferritin iron determinations with a sensitivity limit of 0.4 µM. The coefficient of variation of the different determinations of the same sample was approximately 10%. Total protein was measured according to the method of Lowry et al. (19).

Measurement of ferritin iron saturation

The levels of iron saturation of ferritin in synovial fluids and synovial membranes were calculated using the ferritin concentration value and the ferritin-bound iron concentration value. Three pieces of the same synovial membrane were homogenized separately, and the ferritin iron saturation was determined. This procedure was used with 3 biopsy specimens. With these data, the coefficient of variation of the entire procedure was calculated to be 7%.

Statistical analysis

Because the data showed an asymmetrical distribution, significance was assessed by Wilcoxon rank sum test.

## RESULTS

### FERRITIN LEVELS IN SYNOVIAL FLUID

The ferritin concentration was measured in synovial fluid from 28 patients with RA, 12 with OA, 10 with traumatic knee lesions, 2 with systemic lupus erythematosus (SLE), 4 with psoriasis, and 4 patients with gout (figure 1). The mean ferritin concentration was 5.4 times higher in the synovial fluid of RA patients compared with both patient control groups (knee trauma and OA) ($P < 0.01$), but there was considerable overlap. In the 2 SLE patients, levels of ferritin were within the same range as those found in the RA patients. The psoriasis and gout patients showed values equal to those of the OA patients and the patients with traumatic knee lesions.

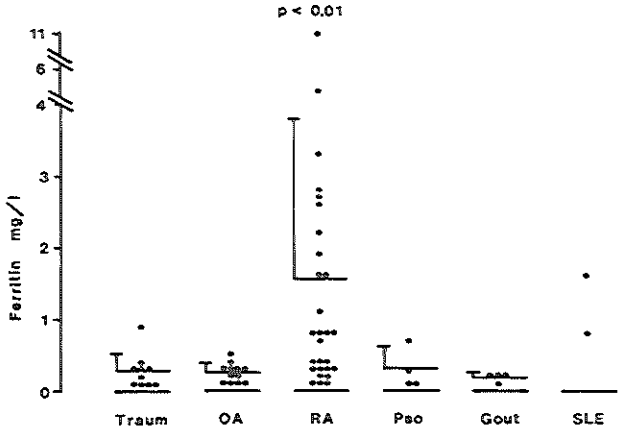



Fig.1: Ferritin concentration in synovial fluids from patients with traumatic knee lesions (Traum), osteoarthritis (OA), rheumatoid arthritis (RA), psoriasis (Pso), gout and systemic lupus erythematosus (SLE). The ferritin level is significantly higher in RA patients versus the group with OA and the group with traumatic knee lesions. Bars show the means + SD.

## SYNOVIAL FLUID: SERUM RATIO OF FERRITIN

Ferritin was estimated in paired samples of synovial fluid and serum from 13 RA patients, 8 OA patients, and 3 patients with traumatic knee lesions, to investigate the distribution of ferritin (table 1). In contrast to most other synovial fluid proteins, which show synovial fluid: serum ratios below 1.0, ferritin was found in greater amounts in synovial fluid than in serum. In RA patients, the ratio reached 14.7; this was significantly higher ($P < 0.01$) than the ratio noted in OA patients. Only 3 paired samples could be obtained from the 10 patients with traumatic knee lesions. Although no statistical conclusions can be drawn from so few data, the results suggest that the synovial fluid: serum ferritin ratio in this group is equal to that found in OA patients.

Table 1. Distribution of ferritin in serum and synovial fluid of rheumatoid arthritis (RA) patients and control subjects*.

| | Synovial fluid | Serum | Synovial fluid:serum ratio |
|---|---|---|---|
| RA patients (n=13) | 1.54 ± 2.27 | 0.15 ± 0.25 | 14.7 ± 14.6@ |
| OA patients (control) (n=8) | 0.25 ± 0.13 | 0.12 ± 0.09 | 3.6 ± 3.8 |
| Trauma patients | 0.29 | 0.02 | 13.3 |
| (control) | 0.39 | 0.08 | 4.7 |
| (n=3) | 0.30 | 0.11 | 2.6 |

* Values are mean ± SD (except for the trauma patients whose values are listed separately) mg/l. OA = osteoarthritis; trauma = patients with traumatic knee lesions.

@ $P < 0.01$ versus OA patient group, by Wilcoxon rank sum test.

FERRITIN LEVELS IN SYNOVIAL MEMBRANE

The synovial membranes of 18 patients with RA and 21 patients with traumatic knee lesions were homogenized, and levels of ferritin and total protein were measured. The results, shown in figure 2, reveal no significant difference in ferritin concentrations per gram of protein.

AMOUNT OF FERRITIN-BOUND IRON

The amounts of iron bound to ferritin in the synovial membranes from 12 RA patients and from 10 patients with traumatic knee lesions are shown in figure 3. The mean value in RA patients was 2.9 times higher than that in the control group. Despite the wide range of values in the RA patients, the difference in the mean levels was significant ($P < 0.05$).

Synovial fluid from 13 RA patients contained ferritin-bound iron

at a concentration of 1.8 ± 1.5 µM (mean ± SD). Only a few control specimens could be analyzed since many of the synovial fluid samples were excluded because of low ferritin content. In OA patient samples, the concentration of iron bound to ferritin was in the same range as that in the RA patients, in patients with traumatic knee lesions, however, the concentration tended to be lower.

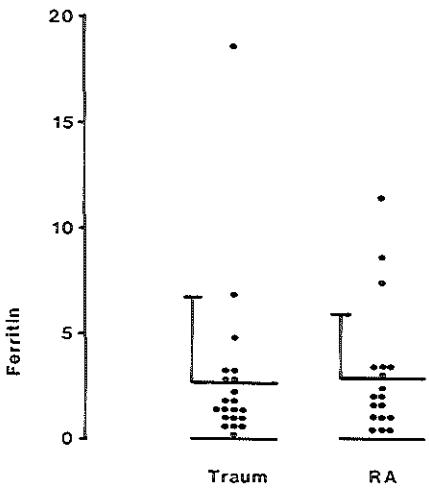



Fig. 2: Ferritin in the synovial membranes from patients with traumatic knee lesions (Traum) and patients with rheumatoid arthritis (RA). The ferritin concentrations are expressed in mg/gm of protein homogenate; bars show the means + SD.

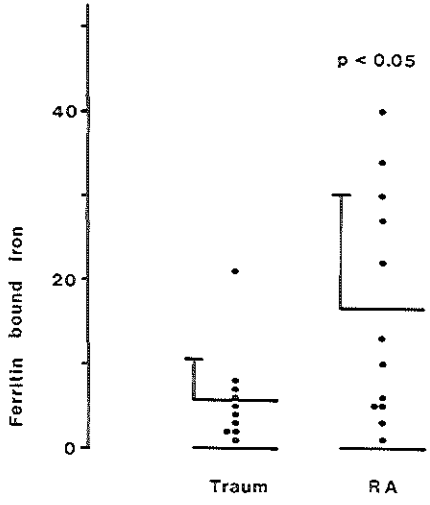



Fig. 3: The concentration of iron bound to ferritin in the synovial membranes from patients with traumatic knee lesions (Traum) and patients with rheumatoid arthritis (RA). The ferritin-bound iron concentrations are expressed in µmoles/gm of protein homogenate; bars show the means + SD.

FERRITIN IRON SATURATION

By using the ferritin values and the iron bound to ferritin values, the iron saturation of ferritin could be calculated. The mean ferritin iron loading in the synovial membranes from patients with traumatic knee lesions (n=10) was 1,500 moles of iron per mole of ferritin, which corresponds with 33% iron saturation of the ferritin molecule (figure 4). The ferritin iron load was significantly higher ($P < 0.01$) in RA patients (n=12), giving a mean value of 2,210 moles/mole (saturation 49%), which is 1.5 times greater than that of the controls.

Of the 10 synovial fluid samples from RA patients, 2 samples were excluded because the ferritin concentrations did not reach the 200 µg/l limit of our detection method. Two other samples contained enough ferritin, but the iron content of ferritin was below the 0.4 µM limit of detection. The saturation in these samples were included in the calculations (assuming that the iron content was 0.4 µM), the true mean values could be considerably lower. These two samples are indicated by the open triangles in figure 4. Synovial fluid from RA patients showed ferritin iron saturation of 1,150 moles/mole, which is about

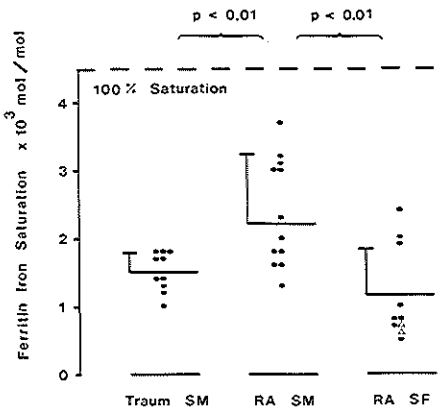



Fig.4: Ferritin iron saturation in the synovial membranes(SM) from patients with traumatic knee lesions (Traum) and patients with rheumatoid arthritis (RA) and the ferritin iron saturation in RA synovial fluids (SF). Bars show the means + SD. Open triangles represent the 2 samples with levels of ferritin-bound iron below the 0.4 µM limit of detection.

half the level found in RA synovial membranes ($P < 0.01$). Synovial fluid from controls was also analyzed, but many samples were excluded because the amount of ferritin was too low. In two patients with traumatic knee lesions, ferritin iron loading was 1,850 moles/mole and 3,280 moles/mole, respectively. Two OA patients had values of 3,000 moles/mole and 1,000 moles/mole, respectively, in their synovial fluid samples. These limited values suggest that, in RA patients synovial fluid ferritin iron loading is decreased in comparison with levels found in controls.

## DISCUSSION

Stimulated granulocytes and macrophages produce, in addition to other inflammatory mediators, large amounts of $O_2^-$ and $H_2O_2$ (7). Investigations during the last decade have shown that these oxygen free radicals play an important role in tissue destruction in RA (3-6). Iron is an essential part of hemoglobin, as well as a variety of proteins and enzymes in the body. However, iron is very toxic because it enhances the toxicity of oxygen free radicals (20-22). Rather inactive reactive oxygen species, such as $O_2^-$ and $H_2O_2$, are converted to extremely toxic species, such as the hydroxyl radical, in the presence of non-protein-bound iron.

Data from several investigations suggest an important role for iron in RA. Levels of total iron are increased in RA synovial fluid and synovial membrane in comparison with levels found in controls (1, 23). Secondly, arthritis is frequently found in patients with hemochromatosis and hemophilia, diseases in which iron accumulates in joints. Moreover, RA patients receiving iron intravenously experienced an exacerbation of their arthritis (2).

The iron-binding proteins, transferrin and lactoferrin, protect against free radical attack by binding iron in a form that is unable to catalyze hydroxyl radical formation (9). Although transferrin is present in high concentrations in synovial fluid (24), it does not protect, because as a result of oxygen free radical damage, it has lost its capacity to bind iron (16). Ferritin also protects against iron-catalyzed free radical damage, but only partially. Ferritin is able to increase free radical damage (25, 26), although at a lower

rate than corresponding amounts of free iron (10). The differences between ferritin and transferrin or between ferritin and lactoferrin have been explained by previous studies that have shown that superoxide is able to release iron from ferritin only (13).

The present study focused on the amount of ferritin, and more importantly, the amount of iron bound to ferritin, that is present intra-articularly in RA patients. We sought to determine whether iron is available to catalyze the conversion of $O_2^-$ and $H_2O_2$ to the very toxic $OH^\cdot$ radical. The ferritin concentration in synovial fluid was 5.4 times higher in RA patients compared with that in OA patients and in patients with traumatic knee lesions. Our results confirm the results of previous investigations (14).

Measurements of the ferritin concentrations in paired samples of synovial fluid and serum revealed that in RA, ferritin is much higher in synovial fluid than in serum. In controls, this ratio was significantly lower, although still considerably higher than 1.0. In contrast, Milman et al. (27) found the same ferritin concentration ratios in serum and synovial fluid samples from RA patients and OA patients. Almost all serum proteins can be found in synovial fluid, but at concentrations below those in serum (28). A ratio above 1.0 suggests that a protein is produced locally and is not derived from serum. The ferritin present in synovial fluid most likely originates from synthesis in synovial membrane cells, which when they die, release the ferritin into the joint cavity. Previous studies using microscopy showed ferritin to be present in the synovial membrane of RA patients; however, only qualitative information was obtained in the studies, and no control group was included (29, 30). We measured ferritin quantitatively. Equal amounts of ferritin (per gram of protein homogenate) were detected in the syovial membranes of patients with RA and patients with traumatic knee lesions. Knowing that the total volume of synovial membrane tissue is increased in RA, the ferritin content of an entire joint will naturally be higher in RA patients than in controls.

The potential danger of ferritin is related to its iron content. Blake et al. (16) reported that the ferritin found in synovial fluid contained no, or hardly any, iron. Our results do not support this finding. While RA synovial fluid was clearly shown to contain micro-

molar amounts of ferritin-bound iron, the limited data from control specimens suggested that, in patients with traumatic knee lesions, the ferritin-bound iron concentration in synovial fluid is less than that found in RA patients. Considerable amounts of ferritin-bound iron were found in the synovial membranes of RA patients, and the levels were statistically significantly higher than those found in specimens from the controls. These results clearly show that in RA, ferritin-bound iron is present in concentrations that are adequate to stimulate the hydroxyl radical formation.

The increase in iron bound to ferritin in RA patients can be explained by two mechanisms that are possibly of equal importance.

1. In RA, repeated lesions of the inflamed, highly vascular synovial membrane occur, and these result in small hemorrhages. It has been suggested that there is a total intra-articular blood loss of 4 ml in 24 hours (31). Under such conditions, erythrocytes will be destroyed and their iron will accumulate in the synovial membrane cells, and ferritin will be produced (32).
2. RA is complicated by an increased affinity of macrophages for iron, which results in little iron being available for hemoglobin synthesis. Macrophage-derived cells are a major component of the synovial membrane. Thus, by this mechanism, considerable amounts of serum-derived iron can be sequestrated into the synovial membrane.

Measurements of ferritin and iron bound to ferritin in the same tissue samples made in possible to calculate the ferritin iron saturation. RA synovial membranes had a mean iron saturation of 2,210 moles of iron per mole of ferritin, which was significantly elevated compared with the value of 1,500 moles/mole found in controls. RA synovial fluid contained ferritin with a mean iron saturation of 1,150 moles/mole. This result contrasts with the observation that synovial fluid ferritin does not contain iron (16). Our method of the chemical measurement of ferritin-bound iron is superior to the density gradient centrifugation method used in that study (16). A comparison of ferritin iron saturation in synovial membrane versus synovial fluid in RA patients revealed a significant decrease in saturation in synovial fluid. If synovial fluid ferritin is derived from the synovial membrane, similar levels of iron saturation are expected. The decreased saturation can be explained by iron mobilization induced by stimulated

granulocytes, as shown previously (13). Most likey, iron mobilization from ferritin also occurs in the synovial membrane, but this can be compensated for by de novo synthesis of ferritin, and the steady state saturation level will not fall.

Our results support the hypothesis about tissue destruction in RA that is illustrated in figure 5. Superoxide from stimulated granulocytes and macrophages will mobilize iron from ferritin, which is present in sufficient amounts in both synovial membrane and synovial fluid. This iron is available to convert $O_2^-$ and $H_2O_2$, derived from the inflammatory cells, to the hydroxyl radical. The hydroxyl radical is able to destroy cartilage, membranes, hyaluronic acid, and proteins.

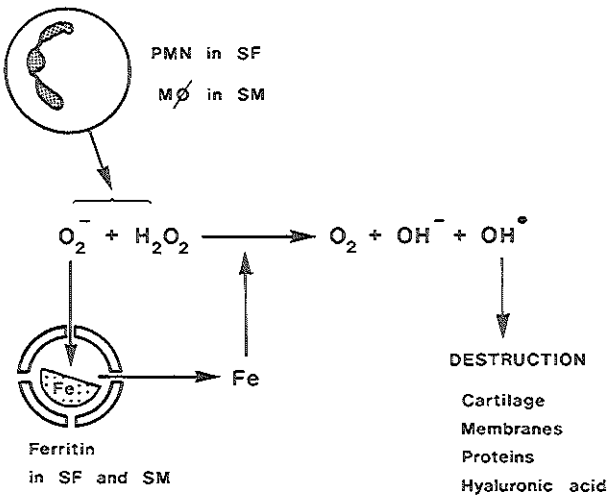



Fig.5: Hypothesis regarding the role of ferritin in the pathogenesis of rheumatoid arthritis. PMN= polymorphonuclear cell; SF= synovial fluid; MØ= macrophage; SM= synovial membrane.

ACKNOWLEDGEMENTS

The authors thank C.M. Beindorff and M.J. Kroos for their excellent technical support, I Simon for obtaining the tissue samples from rheumatoid arthritis and osteoarthritis patients, Dr. M.P. Heyboer for the samples from patients with traumatic knee lesions, Dr. F.M.J. Zuyderhoudt for the fruitful discussion, Dr. G.R. Elliott for correction of the English language, and A.I. Leusink for preparing the manuscript.

REFERENCES

1. Mowat AG, Hothersall TE: Nature of anaemia in rheumatoid arthritis. VIII. Iron content of synovial tissue in patients with rheumatoid arthritis and in normal individuals. Ann Rheum Dis 27:345, 1968.
2. Blake DR, Lunec J, Ahern M, Ring EFJ, Bradfield J, Gutteridge JMC: Effect of intravenous iron dextran on rheumatoid synovitis. Ann Rheum Dis 44:183, 1985.
3. Greenwald RA, Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum 23:455, 1980.
4. Lunec J, Halloran SP, White AG, Dormandy TL: Free-radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J Rheumatol 8:233, 1981.
5. Fantone JC, Ward PA: Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am J Pathol 107:395, 1982.
6. Schalkwijk J, Van den Berg WB, Van de Putte LBA, Joosten LAB: Hydrogen peroxide suppresses the proteoglycan synthesis of intact articular cartilage. J Rheumatol 12:205, 1985.
7. Babior BM, Kipness RS, Curnutte JT: Biological defense mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741, 1973.
8. Gutteridge JMC, Rowley DA, Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: detection of "free" iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199:263, 1981.

9. Baldwin DA, Jenny ER, Aisen P: The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J Biol Chem 259:13391, 1984.
10. Gutteridge JMC, Halliwell B, Treffry A, Harrison PM, Blake D: Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem J 209:557, 1983.
11. Crichton RR: Structure and function of ferritin. Angew Chem Int Ed Engl 12:57, 1973.
12. Harrison PM: Ferritin: an iron-storage molecule. Semin Hematol 14:55, 1977.
13. Biemond P, Van Eijk HG, Swaak AJG, Koster JF: Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes: Possible mechanism in inflammation diseases. J Clin Invest 73:1576, 1984.
14. Blake DR, Bacon PA, Eastham EJ, Brigham K: Synovial fluid ferritin in rheumatoid arthritis. Brit Med J 281:715, 1980.
15. Blake DR, Hall ND, Bacon PA, Dieppe PA, Halliwell B, Gutteridge JMC: The importance of iron in rheumatoid disease. Lancet ii:1142, 1981.
16. Blake DR, Brailsford S, Winyard P, Lunec J: A role for extracellular ferritin in inflammation (abstract). Ann Rheum Dis 43:107, 1984
17. Ropes MW, Bennett GA, Cobb S, Jacox R, Jessar RA: 1958 revision of diagnostic criteria for rheumatoid arthritis. Bull Rheum Dis 9:175, 1958.
18. Zuyderhoudt FJM, Linthorst C, Hengeveld P: On the iron content of human serum ferritin, especially in acute viral hepatitis and iron overload. Clin Chim Acta 90:93, 1978.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurements with the folin phenol reagents. J Biol Chem 193:265, 1951.
20. Halliwell B, Gutteridge JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1, 1984.
21. Graf E, Mahoney JR, Bryant RG, Eaton JW: Iron-catalyzed hydroxyl radical formation: stringent requirement for free iron coordination site. J Biol Chem 259:3620, 1984.
22. Anonymous: Metal chelation therapy, oxygen radicals, and human disease. Lancet ii:143, 1985.

23. Niedermeier W, Griggs JH: Trace metal composition of synovial fluid and blood serum of patients with rheumatoid arthritis. J Chronic Dis 23:527, 1971.
24. Biemond P, Swaak AJG, Koster JF: Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid. Arthritis Rheum 27:760, 1984.
25. Thomas CE, Morehouse LA, Aust SD: Ferritin and superoxide-dependent lipid peroxidation. J Biol Chem 260:3275, 1985.
26. O'Conell MJ, Ward RJ, Baum H, Peters TJ: The role of iron in ferritin- and haemosiderin-mediated lipid peroxidation in liposomes. Biochem J 229:135, 1985.
27. Milman N, Hansen SE, Strandberg Pedersen N: Synovial fluid ferritin in rheumatic diseases. Scand J Rheumatol 14:33, 1985.
28. Kushner I, Somerville JA: Permeability of human synovial membrane to plasma proteins: relationship to molecular size and inflammation. Arthritis Rheum 14:560, 1971.
29. Muirden KD: Ferritin in synovial cells in patients with rheumatoid arthritis. Ann Rheum Dis 25:387, 1966.
30. Blake DR, Gallagher PJ, Potter AR, Bell MJ, Bacon PA: The effect of synovial iron on the progression of rheumatoid disease: a histologic assessment of patients with early rheumatoid synovitis. Arthritis Rheum 27:495, 1984.
31. Bennett RM, Williams ED, Lewis SM, Holt PJL: Synovial iron deposition in rheumatoid arthritis. Arthritis Rheum 16:298, 1973.
32. Muirden KD, Fraser JRE, Clarris B: Ferritin formation by synovial cells exposed to haemoglobin in vitro. Ann Rheum Dis 26:251, 1967.

CHAPTER 6.

# ON THE SUPEROXIDE-DEPENDENT AND INDEPENDENT MECHANISM OF IRON MOBILIZATION FROM FERRITIN BY XANTHINE OXIDASE. ITS IMPLICATIONS FOR OXYGEN FREE RADICAL INDUCED TISSUE DESTRUCTION DURING ISCHEMIA AND INFLAMMATION

P. Biemond, A.J.G. Swaak, C.M. Beindorff and J.F. Koster

Biochem J, in press

KEY WORDS: Xanthine oxidase, xanthine, superoxide, ferritin, oxygen free radicals, iron mobilization, ischemia, inflammation, uric acid.

**ABSTRACT**

Xanthine oxidase is able to mobilize iron from ferritin. This mobilization can be blocked by 70% by superoxide dismutase indicating that part of its action is mediated by superoxide ($O_2^-$). Uric acid induced the release of ferritin iron at concentrations normally found in serum. The $O_2^-$-independent mobilization of ferritin iron by xanthine oxidase cannot be attributed to uric acid, because:

1. uricase did not influence the $O_2^-$-independent part,
2. acetaldehyde, substrate for xanthine oxidase, also revealed a $O_2^-$-independent part, although no uric acid was produced. Presumably the amount of uric acid produced by xanthine oxidase and xanthine is insufficient to release a measurable amount of iron from ferritin.

The liberation of iron from ferritin by xanthine oxidase has important consequences in ischemia and inflammation. In these circumstances xanthine oxidase formed from xanthine dehydrogenase, will stimulate the formation of a non-protein bound iron pool, and the $O_2^-$ produced by xanthine oxidase, or granulocytes, will be converted by "free" iron to much more toxic oxygen species such as hydroxyl radicals ($OH^\cdot$), exacerbating the tissue damage.

**INTRODUCTION**

It has been known for a long time that xanthine oxidase and ferritin interact with each other (1, 2, 3). In the presence and the absence of oxygen (4) xanthine oxidase is able to mobilize iron from ferritin. Mazur et al. (3) suggested that xanthine oxidase was important in mobilization of iron from liver ferritin in vivo. Various studies have provided data both supporting (5, 6) and opposing (7, 8) this hypothesis. Thopham et al. (9) concluded that the proposed hypothesis was true, because they found an accumulation of iron in the liver after inhibition of xanthine oxidase. They suggested that previous negative findings were due to an incomplete inhibition of xan-

thine oxidase.

Ferritin is the main iron storage protein in the body. The physiological mechanism of iron mobilization from ferritin is unclear. A reducing substance is needed to form ferrous iron, subsequently the ferrous iron leaves the ferritin core and must be complexed by a suitable chelator (10-12).

The mechanism by which xanthine oxidase is able to release iron from ferritin was unknown for many years. McCord and Fridovich (13) found that xanthine oxidase was able to produce superoxide ($O_2^-$). Xanthine oxidase is now the most commonly used source of $O_2^-$ in in vitro experiments. It is uncomprehensible that until recently no one suggested $O_2^-$ to be responsible for ferritin iron mobilizaton, while it was known that a one electron reduction is necessary. Our previous investigations demonstrated that ferritin iron could be mobilized by $O_2^-$ derived from granulocytes or solid potassium superoxide (14).

Afterwards Thomas et al. (15) have shown that $O_2^-$ produced by xanthine oxidase is also able to release iron from ferritin. In the present study additional information about the mobilization of iron from ferritin by xanthine oxidase is obtained. The consequences of iron mobilization from ferritin in ischemia and inflammation are discussed.

## MATERIALS AND METHODS

4,7 Diphenyl-1,10-phenanthroline disulfonate acid sodium salt (bathophenanthroline), uric acid, xanthine were obtained from E. Merck, Darmstadt, Federal Republic of Germany. Acetaldehyde from B.D.H. Chemicals, Poole, England. Cytochrome c, horse spleen ferritin (22% iron, 50% saturated), transferrin (70% saturated with iron, removal of free iron by gelfiltration), catalase (65.000 U/mg), superoxide dismutase (SOD) (5000 U/mg) and uricase (6 U/mg) came from C.F. Boehringer & Sons, Mannheim, Federal Republic of Germany. All other reagents are of highest analytical grade. Milk xanthine oxidase was purified from fresh cow-milk by the method of Massey et al. (16).

$O_2^-$ production

Xanthine oxidase was tested for its capacity to produce $O_2^-$. The reaction mixture (total volume 1 ml) contained: xanthine oxidase 0.02 g/l, xanthine 0.2 mM, cytochrome c 30 µM in Tris HCl 0.1 M, pH 7.4. Incubation at 20°C was started by addition of xanthine oxidase. The $O_2^-$ production was calculated from the increase in the absorption at 550 nm, using an extinction coefficient of 21,100 $M^{-1}.cm^{-1}$ for cytochrome c. Cytochrome c reduction slowed down in time, because accumulation of $H_2O_2$ caused reoxidation of cytochrome c, which could be inhibited by catalase. So for proper evaluation of $O_2^-$ production, the initial cytochrome c reduction has to be used.

Ferritin iron mobilization

Iron mobilization from ferritin by xanthine oxidase was measured, by incubation of a mixture containing: xanthine oxidase 0.02 g/l, xanthine 0.2 mM, ferritin 0.5 g/l, bathophenathroline 1 mM in Tris HCl 0.1 M, pH 7.4. A serie of experiments was performed with acetaldehyde 10 mM as substrate instead of xanthine. Additions were performed as indicated in figure legends. Incubation at 20°C was started by the addition of xanthine oxidase. Iron mobilization was calculated from the absorption at 530 nm using an extinction coefficient of 22,140 $M^{-1}.cm^{-1}$ for bathophenanthroline. Release of iron from ferritin by uric acid was tested in the same system by substitution of xanthine oxidase and xanthine by uric acid.

## RESULTS

### FERRITIN IRON MOBILIZATION BY XANTHINE OXIDASE

Incubation of horse spleen ferritin with xanthine oxidase and xanthine resulted in release of iron detected by bathophenanthroline (figure 1, part B). In control experiments without xanthine oxidase, xanthine or ferritin, hardly any iron could be detected. Addition of SOD, able to destroy all $O_2^-$ produced by xanthine oxidase (figure 1, part A), inhibited ferritin iron release by 70%, indicating that 70% of iron mobilization is caused by $O_2^-$. Catalase revealed minimal decrease of iron release. Mannitol and dimethylsulfoxide did not have

a significant effect too (data not shown). It can be concluded that $H_2O_2$ and $OH^{\cdot}$ are not involved in ferritin iron mobilization by xanthine oxidase. Xanthine oxidase was unable to release iron from transferrin (70% iron saturation) 3 g/l in our system.

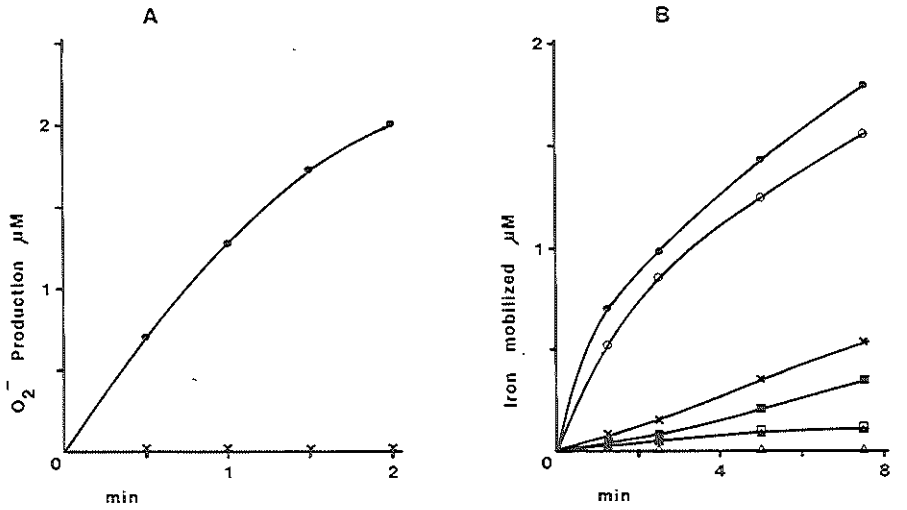



Fig.1: Superoxide production (A) and iron mobilization from ferritin by xanthine oxidase and xanthine (B).
A: Reaction mixture (1ml) contained xanthine oxidase 0.02 g/l, xanthine 0.2 mM cytochrome c 30 µM in Tris HCL 0.1 M, pH 7.4. Absorbance was measured continuously at 550 nm.
B: Reaction mixture (1 ml) contained xanthine oxidase 0.02 g/l, xanthine 0.2 mM, ferritin 0.5 g/l and bathophenanthroline 1 mM in Tris HCL 0.1 M, pH 7.4. Absorbance was measured continuously at 530 nm.
●, No additions; ○, plus catalase 40 mg/l: ×, plus SOD 0.1 g/l; ▣, plus SOD 0.1 g/l and catalase 40 mg/l; ▲, without xanthine oxidase; □, without xanthine; △, without ferritin.

## FERRITIN IRON MOBILIZATION BY URIC ACID

While $O_2^-$ seemed to be responsible for 70% of ferritin iron mobilization by xanthine oxidase, we looked for an additional mechanism of ferritin iron release. FAD or FMN on their own did not reveal iron mobilization from ferritin unless the incubation mixture was illuminated. Uric acid, the product of the reaction between xanthine oxidase and xanthine, was able to release iron from ferritin as shown in figure 2. The amounts depended on the uric acid concentration,

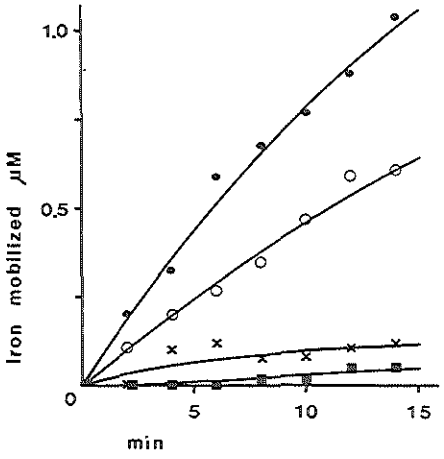



Fig.2: Iron mobilization from ferritin by uric acid. Incubation mixture (1ml) containned ferritin 0.5 g/l, bathophenanthroline 1 mM and uric acid in Tris HCL 0.1 M, pH 7.4 Absorbance was measured at 530 nm. ●,uric acid 200 µM; ○, uric acid 50 µM; ×, no uric acid; ■, uric acid 50 µM plus uricase 10 mg/l.

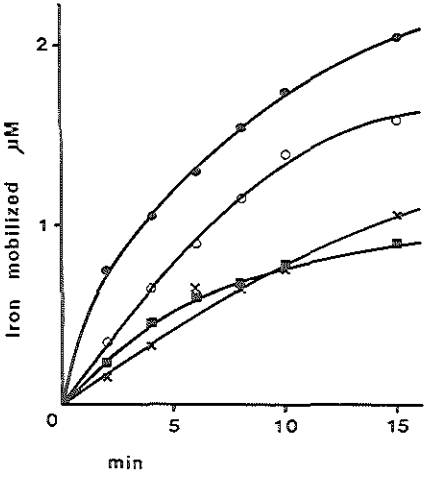



Fig.3: Iron mobilization from ferritin by xanthine oxidase and xanthine. Inhibition by SOD and uricase. Reaction mixture (1ml) contained xanthine oxidase 0.02 g/l, xanthine 0.2 mM, ferritin 0.5 g/l and bathophenanthroline 1 mM in Tris HCL 0.1 M, pH 7.4 Absorbance was measured at 530 nm. ●, no additions; ○, plus uricase 10 mg/l; ×,plus SOD 0.1 g/l; ■, plus uricase 10 mg/l; and SOD 0.1 g/l.

which were within the range normally found in serum. Addition of uricase caused a decreased of iron mobilization. Control experiments excluded a direct interaction between uric acid and bathophenanthroline. The contribution of uric acid in ferritin iron mobilization by xanthine oxidase was investigated by using uricase (figure 3). In the presence of SOD, uricase did not have an additional inhibitory effect on ferritin iron release by xanthine oxidase and xanthine, suggesting that the $O_2^-$-independent part was not due to an effect of uric acid. Uricase without SOD, however, did show a small decrease in iron mobilization. Superoxide production by xanthine oxidase did not change in the presence of uricase. Liberation of iron from ferritin also occurred after replacement of xanthine by acetaldehyde. SOD

inhibited iron release for 80%, which suggested that about 20% of the iron mobilization was $O_2^-$-independent. These various results indicate that although uric acid is able to release iron from ferritin, its action is insufficient to explain the complete $O_2^-$-independent part of ferritin iron release by xanthine oxidase.

## DISCUSSION

In the present study iron mobilization from horse spleen ferritin by xanthine oxidase was found. It could be inhibited by 70% by SOD, at a concentration able to inhibit all $O_2^-$ production by xanthine oxidase (figure 1). In contrast catalase, dimethylsulfoxide (DMSO), and mannitol had no effect. On the basis of the inhibition by SOD it can be concluded that the majority of ferritin iron mobilization was $O_2^-$-dependent, but additionally a $O_2^-$-independent part was found. Thomas et al. (15) found iron mobilization by xanthine oxidase and xanthine, which could be completely blocked by SOD.

Xanthine oxidase was unable to release iron from human transferrin saturated with iron for 70%.

Further investigations were performed to elucidate the mechanism of the $O_2^-$-independent iron mobilization. Uric acid, the product of the reaction between xanthine oxidase and xanthine, did mobilize iron from ferritin, in concentrations which are normally found in serum (figure 2). This mechanism was already suggested by Green et al. (2). Liberation of iron from ferritin by uric acid may increase oxygen free radical damage, which is in sharp contrast to its function as protector against these radicals as suggested by Ames et al. (17). However, their findings could not be reproduced by us (18). To measure the part of ferritin iron mobilization by xanthine oxidase and xanthine caused by uric acid, uricase was used (figure 3). After inhibition by SOD, uricase did not have an additional inhibiting effect on the iron release, indicating that the contribution of uric acid was small or absent. A second argument for uric acid not being responsible for the $O_2^-$-independent part was found by using acetaldehye as a substrate for xanthine oxidase. Iron mobilization from ferritin then was inhibited by SOD by 80%, indicating that 20% of iron mobilization was indepen-

dent of both $O_2^-$ and uric acid. Presumably the amount of uric acid produced by xanthine oxidase is insufficient to cause a measurable amount of ferritin iron mobilization. FAD and FMN are unable to release iron from ferritin without illumination of the incubation mixture.

The $O_2^-$-independent part can possibly be explained by a direct electron transfer from xanthine oxidase to ferritin. However, considering the fact that these proteins are quite large, stereometric problems are to be expected. The presumable interaction between xanthine and ferritin is not strong, because after the incubation the proteins could be easily separated by isoelectric focussing.

Surveying the data we conclude that iron mobilization from ferritin by xanthine oxidase depends on more than one mechanism:

1. $O_2^-$-dependent, responsible for about 70%,
2. $O_2^-$-independent, possibly using and electron carrier different from $O_2^-$ which could not be detected, or by direct electron transfer from the enzyme to ferritin, for example by transfer of electrons via the protein shell to the iron core.

An $O_2^-$-independent mechanism is substantiated by ferritin iron release caused by xanthine oxidase under anaerobic conditions (2, 4). Although ferritin iron mobilization is possible by uric acid, it is negligible in xanthine oxidase ferritin iron mobilization.

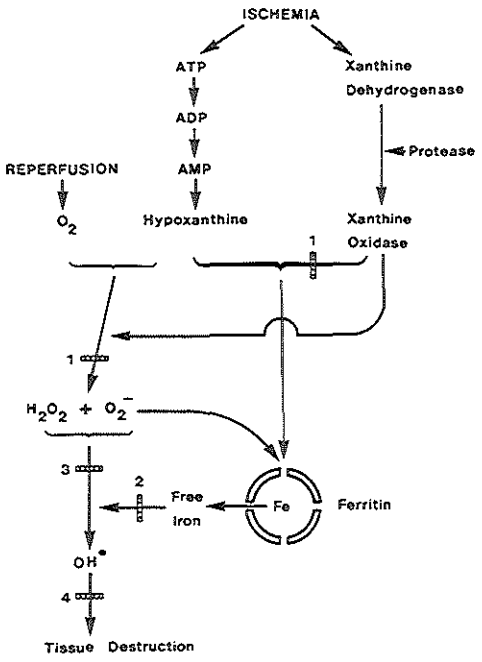
These results have serious implications in oxygen free radical tissue damage. Superoxide is relatively innocent on its own, but its toxicity increases enormously in the presence of "free" iron, due to formation of hydroxyl radicals ($OH^\cdot$), ferryl or perferryl radicals (19-21). Iron present in enzymes, heam-containing proteins or specific iron-binding proteins like transferrin or lactoferrin is not available for the catalysation of $OH^\cdot$ formation (22-25). Ferritin has been shown to stimulate $OH^\cdot$ formation by some workers (26-28) although others found no effect (22). In view of the present results it is very likely that the stimulation of $OH^\cdot$ formation by ferritin can be explained by iron release from ferritin by $O_2^-$, followed by catalysation by "free" iron of $OH^\cdot$ formation.

Evidence is accumulating that oxygen free radicals are involved in the pathogenesis of ischemia. McCord et al. (29, 30) suggested that during ischemia xanthine dehydrogenase, the natural occuring enzyme

which uses $NAD^+$ as alectron acceptor, is converted to xanthine oxidase, which uses oxygen as electron acceptor, resulting in $O_2^-$ formation. Xanthine dehydrogenase is present in considerable amounts in many tissues (31). Simultaneously, during ischemia, ATP is broken down via AMP to hypoxanthine, which together with oxygen, present due to reperfusion or due to partial ischemia, are the substrates necessary for $O_2^-$ production. This hypothesis was supported by the benificial effects of superoxide dismutase during intestinal and myocardial ischemia. In addition, post-ischemic tissue destruction could be inhibited by allopurinol, an inhibitor of xanthine oxidase (32-36).

The potential consequences of ferritin iron mobilization by xanthine oxidase are shown in scheme 1. During ischemia xanthine oxidase will release iron from ferritin. Possibly iron mobilization already starts before reintroduction of oxygen due to the $O_2^-$-independent mechanism. Non-protein bound iron accumulates, able to catalyse the formation of the highly toxic hydroxyl radical, from $O_2^-$ produced by xanthine oxidase. By this serie of events, tissue damage increases dramatically. In ischemic conditions $O_2^-$ can also be produced by granulocytes (37).

The protective effect of allopurinol in ischemia can be explained by prevention of $O_2^-$ production by xanthine oxidase. We suggest that the inhibition of iron release from ferritin by xanthine oxidase is also important in the effect of allopurinol. The conclusion of Chambers et al. (36) that $O_2^-$ production by granulocytes is not of importance in the ischemic myocardium because it can be prevented by allopurinol, is not correct. Allopurinol will block the release of iron from ferritin by xanthine oxidase, free iron will be depressed and $OH^\cdot$ formation from $O_2^-$ produced by granulocytes will diminish. Desferrioxamin is also a potential therapeutic agent in the prevention of ischemic tissue injury on basis of our theory, as well as SOD, catalase, antioxidants and radical scavengers. During inflammation xanthine oxidase could possibly be formed from xanthine dehydrogenase by proteases released from granulocytes, macrophages or dying cells. If this is true, iron mobilization from ferritin by xanthine oxidase could also play a role in a wide spectrum of inflammatory diseases.




Scheme: The role of xanthine oxidase and iron mobilized from ferritin in tissue destruction in ischemia. Inhibitors: 1. allopurinol; 2. desferrioxamin; 3. SOD, catalase; 4. radical scavengers.

ACKNOWLEDGEMENTS

We wish to thank Dr. F. Müller for discussion and isolation of xanthine oxidase; Dr. G.R Elliott for correction of the English language and Mrs. L.P.C. Harkes for preparing the manuscript.

REFERENCES

1. Mazur A, Baez S, Shorr E: The mechanism of iron release from ferritin as related to its biological properties. J Biol Chem 213:147, 1955.
2. Green S, Mazur A: Relation of uric acid metabolism to release of iron from hepatic ferritin. J Biol Chem 227:653, 1957.

3. Mazur A, Green S, Saha A, Carleton A: Mechanism of release of ferritin iron in vivo by xanthine oxidase. J Clin Invest 37:1809, 1958.
4. Duggan DE, Streeter KB: Inhibition of ferritin reduction by pyrazolo[3,4]pyrimidines. Arch Biochem Biophys 156:66, 1973.
5. Mazur A, Carleton A: Hepatic xanthine oxidase and ferritin iron in the developing rat. Blood 26:317, 1965.
6. Powell LW, Emmerson BT: Haemosiderosis associated with xanthine oxidase inhibition. Lancet i:239, 1966.
7. Kinney TD, Kaufman N, Klavins JV: Xanthine oxidase activity and iron storage in the liver. Proc Soc Exp Biol Med 108:22, 1961.
8. Kozma C, Salvador RA, Elion GB: Chronic allopurinol administration and iron storage in mice. Life Sci 7:341, 1968.
9. Topham RW, Walker MC, Calisch MP: Liver xanthine dehydrogenase and iron mobilization. Biochem Biophys Res Commun. 109:1240, 1982.
10. Crichton RR: Structure and function of ferritin. Angew Chem Int Ed Engl 12:57, 1973.
11. Harrison PM: Ferritin: an iron-storage molecule. Semin Hematol 14:55, 1977.
12. Crichton RR, Roman F, Roland F, Paques E, Paques A, Vandamme E: Ferritin iron deposition and mobilization. J Mol Catal 7:267, 1980.
13. McCord JM, Fridovich I: The reduction of cytochrome c by milk xanthine oxidase. J Biol Chem 243:5753, 1968.
14. Biemond P, Van Eijk HG, Swaak AJG, Koster JF: Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. J Clin Invest 73:1576, 1984.
15. Thomas CE, Morehouse LA, Aust SD: Ferritin and superoxide-dependent lipid peroxidation. J Biol Chem 260:3275, 1985.
16. Massey V, Brumby PE, Komai H, Palmer G: Studies on milk xanthine oxidase, some spectral and kinetic properties. J Biol Chem 244:1682, 1969.
17. Ames BN, Cathcart R, Schwiers E, Hochstein P: Uric acid provides an antioxidant defence in humans against oxidant- and radical-caused ageing and cancer: A hypothesis. Proc Natl Acad Sci USA 78:6858, 1981.

18. Koster JF, Slee RG: Lipid peroxidation on human erythrocyte ghosts induced by organic hydroperoxides. Biochim Biophys Acta 752:233, 1983.

19. Aust SD, Svingen BA: The role of iron in enzymatic lipid peroxidation. In: Free Radicals in Biology, vol. 5. Pryor WA (ed) Acad Press, New York, 1, 1982.

20. Graf E, Mahoney JR, Bryant RG, Eaton JW: Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. J Biol Chem 259:3620, 1984.

21. Anonymous: Metal chelation therapy, oxygen radicals and human disease. Lancet i:143, 1985.

22. Gutteridge JMC, Rowley DA, Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of "free" iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199:263, 1981.

23. Winterbourn CC: Lactoferrin-catalysed hydroxyl radical production. Additional requirement for a chelating agent. Biochem J 210:15, 1983.

24. Halliwell B, Gutteridge JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J, 219:1, 1984.

25. Baldwin DA, Jenny ER, Aisen P: The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J Biol Chem 259:13391, 1984.

26. Wills ED: Mechanisms of lipid peroxide formation in animal tissues. Biochem J 99:667, 1966.

27. Gutteridge JMC, Halliwell B, Treffry A, Harrison PM, Blake D: Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem J 209:557, 1983.

28. Carlin G, Djursäter R: Xanthine oxidase induced depolymerization of hyaluronic acid in the presence of ferritin. FEBS Lett 177:27, 1984.

29. Battelli MG, Corte ED, Stirpe F: Xanthine oxidase type D (dehydrogenase) in the intestine and other organs of the rat. Biochem J 126:747, 1972.

30. McCord JM, Roy RS: The pathophysiology of superoxide: Roles in inflammation and ischemia. Can J Physiol Pharmacol 60:1346, 1982.

31. Krenitsky TA, Tuttle JV, Cattau EL, Wang P: A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. Comp Biochem Physiol 49b:687, 1974.
32. Crowell JW, Jones CE, Smith EE: Effect of allopurinol on hemorrhagic shock. Am J Physiol 216:744, 1969.
33. DeWall RA, Vasco KA, Stanley EL, Kezdi P: Responses of the ischemic myocardium to allopurinol. Am J Heart 82:362, 1971.
34. Granger DN, Rutili G, McCord JM: Superoxide radicals in feline intestinal ischemia. Gastroenterol 81:22, 1981.
35. McCord JM: Mechanism of disease: Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312:159, 1985.
36. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM: Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol 17:145, 1985.
37. Romson JL, Hook BG, Hook BS, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR: Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 67:1016, 1983.

CHAPTER 7.

# SUPEROXIDE PRODUCTION BY POLYMORPHONUCLEAR LEUKOCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS: IN VIVO INHIBITION BY THE ANTIRHEUMATIC DRUG PIROXICAM DUE TO INTERFERENCE WITH THE ACTIVATION OF THE NADPH-OXIDASE

P. Biemond, A.J.G. Swaak, J.M.A. Penders, C.M. Beindorff and J.F. Koster

Ann Rheum Dis 45:249, 1986

SUMMARY

The superoxide ($O_2^-$) production of stimulated polymorphonuclear leukocytes is increased in patients with rheumatoid arthritis and osteoarthritis compared with controls. Treatment of these different groups with pharmacological amounts of the non-steroidal anti-inflammatory drug piroxicam in vivo resulted in a decrease of about 25% in $O_2^-$ secretion by isolated granulocytes. In vitro experiments showed that piroxicam inhibits $O_2^-$ production of granulocytes by interference with the stimulation of the NADPH-oxidase. Piroxicam caused diminished $O_2^-$ production of membrane fragments if it was present during the stimulation of the NADPH-oxidase of the intact cells. During the actual $O_2^-$ production of the stimulated membrane fragments piroxicam had no effect. It is concluded that piroxicam is able to inhibit granulocyte $O_2^-$ production by blocking the activation of NADPH-oxidase, which results in diminished tissue destruction by oxygen free radicals in inflammatory diseases.

KEY WORDS: granulocyte inhibition, mechanism of NSAIDs, granulocyte membrane fragments, superoxide anion, phagocytosis.

INTRODUCTION

The importance of oxygen free radicals and related activated oxygen intermediates in the pathogenesis of rheumatoid arthritis (RA) is increasingly recognised (1-4). In RA and in other inflammatory diseases polymorphonuclear leukocytes (PMN) and macrophages are stimulated, which results in the secretion of inflammatory mediators, including large amounts of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (5). These cells produce $O_2^-$ by the NADPH-oxidase, an enzyme complex located in the plasma membrane and phagosome. The reduction equivalents to reduce oxygen to $O_2^-$ are derived from NADPH. In the non-phagocytosing cell the NADPH-oxidase is inactive. When the cell is stimulated to phagocytose an activation mechanism for NADPH-oxidase is triggered. Little is known about the molecular basis of this activation mechanism. These activated oxygen intermediates together with secondary formed radicals, like the hydroxyl radical ($OH^\cdot$), are able

to destroy membrane lipids, proteins, deoxyribonucleic acid, hyaluronic acid, and cartilage (3, 6, 7). Previous investigations have shown that different antirheumatic drugs are able to inhibit the production of oxygen free radicals (8-10). Thus it is reasonable to believe that non-steroidal anti-inflammatory drugs (NSAIDs) have a beneficial effect not only by the inhibition of cyclo-oxygenase, as shown by the excellent work of Vane (11), but also by preventing the formation of oxygen free radicals.

Until now the inhibition of the $O_2^-$ producing NADPH-oxidase by NSAIDs has been mostly tested in vitro. This study is focused on the in vivo inhibition of $O_2^-$ production from PMN by piroxicam, which was chosen because of the encouraging results already obtained (12-14). $O_2^-$ production of PMN was tested before and at different times after the administration of piroxicam to patients with RA and osteoarthritis, and to controls. Attention was also given to the molecular level at which piroxicam interferes with the NADPH-oxidase.

## PATIENTS AND METHODS

### Patients

PMN were isolated at different times from patients with classical or definite RA, from patients with osteoarthritis, and from healthy volunteers. Patients on D-penicillamine, gold salts, or corticosteroids were excluded. All other drugs were discontinued three days before administering piroxicam. If necessary paracetamol was allowed.

Piroxicam was given orally, 40 mg at time zero and another 20 mg 24 h later. When blood was taken for PMN isolation a serum sample was also drawn, and the serum piroxicam was measured by high performance thin layer chromatography (15). Before and during the observation period no other NSAIDs were taken.

### PMN isolation

PMN were harvested from defibrinated blood by sodium metrizoate-Ficoll centrifugation (Lymphoprep., Nyegaard & Co., Oslo, Norway). Erythrocytes were lysed by a cold isotonic $NH_4Cl$ solution (16). The final cell suspension contained 95% PMN. At least 95% of the cells were viable, based on the exclusion of trypan blue.

Capacity of PMN to produce $O_2^-$

Isolated PMN were measured for their capacity to produce $O_2^-$ by the cytochrome c reduction method. The isolated cells were resuspended in a solution containing: 137 mM NACl, 5.4 mM KCl, 0.7 mM $NaH_2PO_4$, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5.5 mM glucose, 25 mM trometamol (TRIS) (pH 7.4 at 37°C), and 5 g/l of bovine serum albumin. The incubation mixture contained in final concentrations: $0.7 \times 10^6$ PMN/ml ($0.7 \times 10^9$/l) exactly, 150 µM cytochrome c, and as stimulus 100 µg/l phorbol myristate acetate (PMA) or 1 g/l serum treated zymosan. After 15 min at 37°C absorption at 550 nm was measured. The $O_2^-$ production was calculated from the increase of the absorption by using an extinction coefficient of 21,100 litre $mol^{-1}cm^{-1}$ for cytochrome c. Control experiments showed that cytochrome c reduction was completely blocked by the addition of 13 mg/l superoxide dismutase.

For measurements of the effect of piroxicam *in vitro* PMN were obtained from healthy individuals. Cells were resuspended in: 138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.6 mM $CaCl_2$, pH 7.4 (PiCM buffer). Incubations were performed with $3.0 \times 10^6$ PMN/ml ($3.0 \times 10^9$/l). Piroxicam (Pfizer BV Rotterdam, The Netherlands) was dissolved in 1 M NaOH, followed by correction of the pH, to final concentrations of 25, 50, and 100 µmol/l. After 5 min preincubation (37°C) 150 µM cytochrome c and 200 µg/l PMA were added (final concentrations). At different times samples were drawn and $O_2^-$ production was measured.

$O_2^-$ production by membrane fragments

Membrane fragments were isolated from control persons according to Tauber and Goetzl (17). Isolated PMN were preincubated with or without 100 µM piroxicam for 5 min. This was followed by stimulation of the cells by incubation with 1 mg/l PMA for three minutes at 37°C. Cells were homogenised at 0°C and differential centrifugation followed. $O_2^-$ production was measured in a mixture containing: PMN membrane fragments 10 µg protein/ml (mg/l) in PiCM buffer, 1 mM NADPH, and 150 µM cytochrome C. The absorbance was measured at 550 nm in a double beam spectrophotometer. The effect of the addition of 100 µM piroxicam during the $O_2^-$ production was also studied. All measurements were duplicated.

## RESULTS

### $O_2^-$ PRODUCTION OF PMN ISOLATED FROM PATIENTS WITH UNTREATED RA AND OSTEOARTHRITIS, AND FROM CONTROLS.

At two different times with an interval of 24 h PMN were isolated from blood from patients with RA and osteoarthritis, and from healthy controls, before treatment with piroxicam. Without stimulation of the cells only very small amounts of $O_2^-$ were secreted, without difference between the groups. The $O_2^-$ production after stimulation with either PMA or serum treated zymosan is presented in figure 1. The mean production of the two different PMN isolations from each individual is shown. After stimulation with PMA no significant difference in $O_2^-$ production was found between RA patients and controls. In contrast, patients with osteoarthritis produced 129% $O_2^-$ in comparison with controls ($P < 0.05$). After stimulation with serum treated zymosan $O_2^-$ secretion was significantly higher in both RA and osteoarthritis patients and equalled 212% ($P < 0.02$) and 154% ($P < 0.02$) respectively.

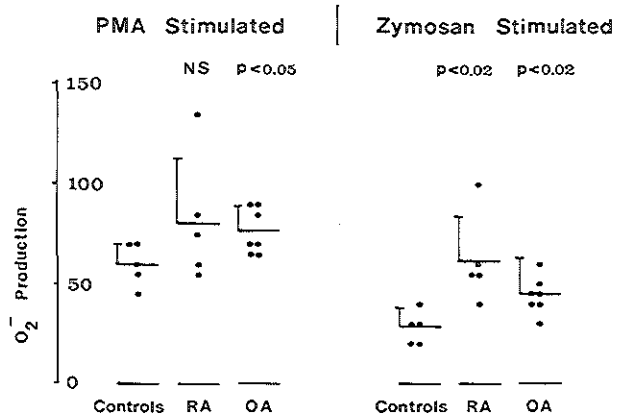



Fig.1: $O_2^-$ production by stimulated PMN from: controls (n=5), patients with rheumatoid arthritis (RA) (n=5), and patients with osteoarthritis (OA) (n=7). Mean and SD of $O_2^-$ production are expressed as nmol/$10^6$ PMN in 15 minutes. PMA or serum treated zymosan was used as stimulus. Significant differences with the control group are indicated. NS= not significant.

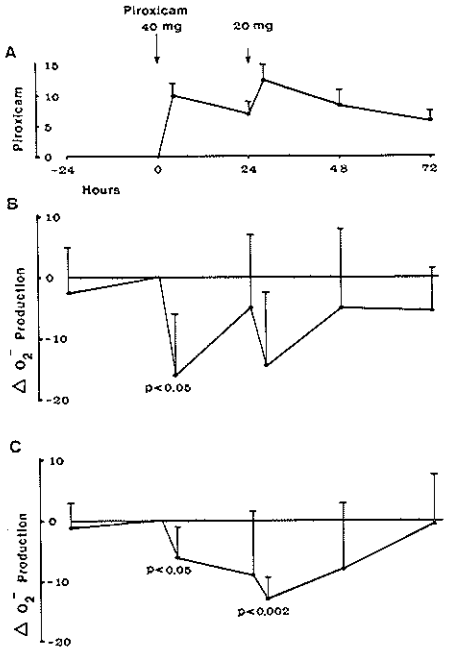



Fig. 2: The effect of piroxicam administered to control persons (n=5) on the $O_2^-$ production by stimulated PMN isolated at different times. (A) Piroxicam concentration in µmol/l; (B) $O_2^-$ production of PMN stimulated with PMA; (C) $O_2^-$ production of PMN stimulated with serum treated zymosan. $O_2^-$ production (in nmol/$10^6$ PMN in 15 minutes) is expressed as the difference in the $O_2^-$ production at the indicated time and at time zero. Mean and SD are shown. Significant differences are indicated.

## $O_2^-$ PRODUCTION OF PMN ISOLATED AFTER TREATMENT WITH PIROXICAM IN VIVO

The effect of administering piroxicam orally on the $O_2^-$ production of PMN isolated after treatment was measured in RA and osteoarthritis patients and in healthy controls. Piroxicam was taken orally at two times, 40 mg at time zero and another 20 mg 24 h later. At the times indicated PMN were isolated and $O_2^-$ production after stimulation with PMA or serum treated zymosan was determined (figures 2, 3 and 4). After taking the blood samples PMN were washed five times so that no serum containing piroxicam was left during the $O_2^-$ estimation. The isolation procedure took about 2 1/2 h. For each individual the difference in $O_2^-$ production at various times was expressed in comparison

with the $O_2^-$ production at time zero, immediately before the first dose of piroxicam. This presentation was used to diminish the effect of variation in $O_2^-$ production before piroxicam treatment.

There was again very little $O_2^-$ production without stimulation of the cells with PMA or serum treated zymosan and this was not changed by piroxicam treatment (data not shown). In the control group (n=5) (figure 2) there was a significant decrease in $O_2^-$ production four hours after taking 40 mg piroxicam, which was independent of PMA or serum treated zymosan as stimulus. At 28 hours, four hours after the second dose of piroxicam (20 mg), the $O_2^-$ production was only significantly decreased when serum treated zymosan was used as stimulus. At 48 and 72 hours the $O_2^-$ production approached the level before treatment. The data at t = -24 hours indicate the variation of the $O_2^-$ production without treatment.

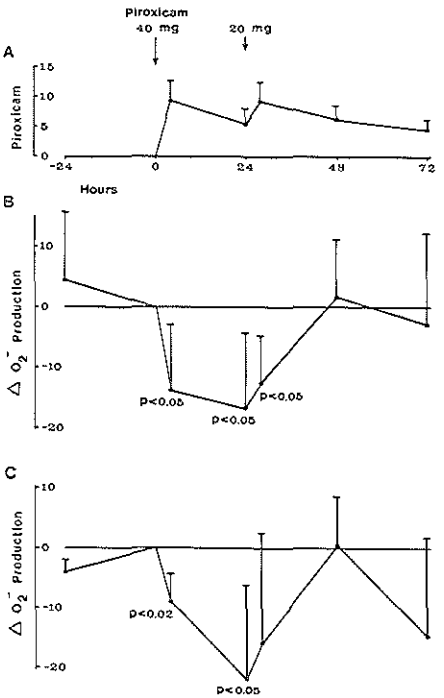



Fig.3: The effect of piroxicam administered to RA patients (n=5) on the $O_2^-$ production by stimulated PMN isolated at different times. For further explanation see legend to Fig. 2.

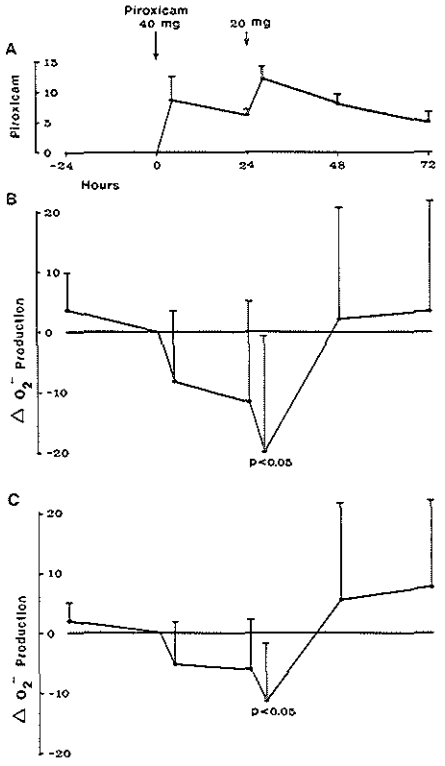



Fig.4: The effect of piroxicam administered to osteoarthritis patients(n=7) on the $O_2^-$ production by stimulated PMN isolated at different times. For further explanation see legend to Fig.2.

The RA patients (n=5) (figure 3) showed similar results. Again piroxicam treatment induced a significant decrease in $O_2^-$ production between four and 28 hours, which was followed by a return to the basal $O_2^-$ production at 48 hours. Patients with osteoarthritis (n=7) (figure 4) showed the same pattern, but the decrease in $O_2^-$ production was significant only at 28 hours.

No significant change in $O_2^-$ production was found in controls without piroxicam treatment in a period of four days, and no diurnal variation was found.

The effect of piroxicam was essentially the same in all different groups. Treatment with piroxicam resulted in an average decrease in $O_2^-$ production of 25%. It should be noted that owing to the extensive washing during the cell isolation, no serum containing piroxicam was

left during the $O_2^-$ measurements. It can be concluded that piroxicam treatment <u>in vivo</u> changed the ability of isolated PMN to produce $O_2^-$.

LEVEL OF INTERACTION OF PIROXICAM WITH THE NADPH-OXIDASE OF PMN

Figure 5 shows that the inhibition of $O_2^-$ production of intact PMN by piroxicam was dependent on concentration. To identify the mode of inhibition of the $O_2^-$ producing NADPH-oxidase complex PMN were homogenised after stimulation of the NADPH-oxidase with PMA. The capacity of membrane fragments to produce $O_2^-$ was not changed by the addition of 100 µM piroxicam during the $O_2^-$ production (figure 6). Membrane fragments obtained from PMN stimulated with PMA in the presence of piroxicam produced smaller amounts of $O_2^-$ in comparison with fragments of cells stimulated in the absence of piroxicam. Addition of piroxicam to these membrane fragments did not result in enhanced

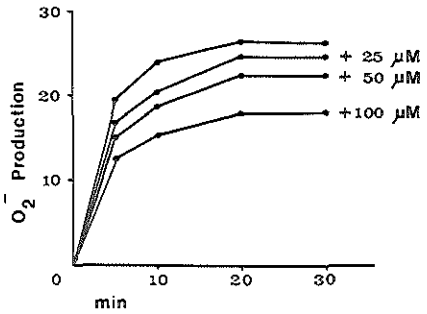



Fig.5: The in vitro inhibition of $O_2^-$ production (nmol/$10^6$ PMN in 15 minutes) of PMN stimulated by PMA incubated in presence of various piroxicam concentrations.

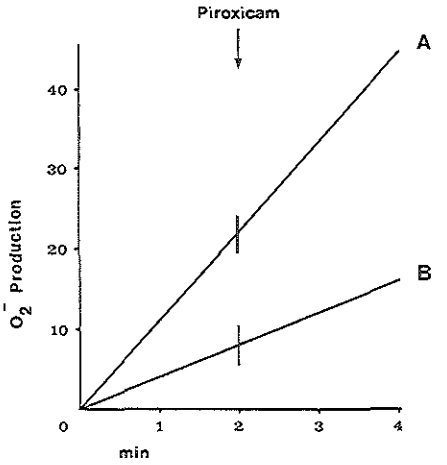



Fig.6: $O_2^-$ production (nmol/mg protein) by membrane fragments of PMN stimulated by PMA. During stimulation of the NADPH-oxidase of the intact PMN 100 µM piroxicam was absent in A and present in B. The effect of 100 µM piroxicam during the $O_2^-$ production is also shown (addition indicated by an arrow).

inhibition. These results indicate that piroxicam has no effect on the NADPH-oxidase complex itself if it is already stimulated, but that piroxicam interferes in the mechanism by which the NADPH-oxidase is stimulated.

## DISCUSSION

The exact role of oxygen free radicals in the pathogenesis of RA is not yet established. However, many authors are convinced that these radicals have an important role (1-4). Phagocytosing granulocytes are known to produce considerable amounts of $O_2^-$ and $H_2O_2$ (5). In juvenile RA stimulated granulocytes are shown to produce twice as much $O_2^-$ as control PMN (18). No difference in $O_2^-$ production was found between adult RA patients and controls (17) but the study of Chiu et al. showed that PMN from patients with Felty's syndrome produced only 63% $O_2^-$ compared with the controls (19). The present investigations show that after stimulation with serum treated zymosan PMN from patients with RA or osteoarthritis produce an increased amount of $O_2^-$ compared with controls. With PMA stimulated PMN the increase was only significant in the osteoarthritis group. This increased potency of PMN to produce $O_2^-$ together with the availability of stimulating factors for PMN in serum and synovial fluid of RA patients (20) will result in considerable $O_2^-$ production *in vivo*. In the presence of non-protein bound iron, possibly derived from ferritin (21), formation of the very toxic hydroxyl radical has to be expected, and tissue destruction will result.

The discovery of the inhibition of prostaglandin synthesis by NSAIDs, due to inhibition of cyclo-oxygenase, indicated an important mechanism of action of NSAIDs. At this moment, however, it seems very likely that in addition other mechanisms have a role in the beneficial effect of these drugs. Previous investigations have shown that NSAIDs like indomethacin, ibuprofen, phenylbutazone and piroxicam (8-12) are able *in vitro* to decrease the release of $O_2^-$ from PMN. Abramson et al. (13, 14) showed that piroxicam did also inhibit $O_2^-$ production of PMN *in vivo* in healthy volunteers. In four RA patients no significant

effect was found (22). In these studies $O_2^-$ production was measured only at two times - before and during treatment with piroxicam. The present study shows the effect of two doses of piroxicam, and $O_2^-$ production was measured at seven different times - twice before treatment to show the natural variation, at different times during the phase of high piroxicam serum levels, and during the recovery phase.

The results show that treatment of patients with piroxicam caused diminished $O_2^-$ production of their PMN. The average decrease was 25%. In contrast with the studies of Abramson et al. (13, 14, 22), we found not only significant decrease in $O_2^-$ production in normals but also in RA and osteoarthritis patients. For the different groups: RA patients, osteoarthritis patients, and controls, no essential differences in the inhibition patterns were found. During the recovery phase the $O_2^-$ production showed a tendency to normalise. In vivo PMN probably are inhibited to a larger extent, because the isolation procedure of the PMN lasted for some time and involved frequent washing of the cells. These manipulations will possible decrease the effect of piroxicam on the cells. The conclusion can be drawn that in vivo piroxicam is able to decrease the $O_2^-$ production of PMN to a considerable extent.

To obtain information about the mechanism of inhibition investigations were performed on the level at which piroxicam inhibited the $O_2^-$ production of PMN. Isolated membrane fragments were used, which are known to contain the $O_2^-$ producing NADPH-oxidase. Stimulation of the NADPH-oxidase is only possible in intact cells, possibly with the exception of stimulation with cis unsaturated fatty acids (23). For this reason cells were homogenised after stimulation of the NADPH-oxidase with PMA, followed by isolation of the membrane fragments. The $O_2^-$ production of the PMA stimulated NADPH-oxidase of membrane fragments decreased if piroxicam was present during the activation of the NADPH-oxidase in the intact cells. Addition of piroxicam to the isolated membrane fragments had no effect on the $O_2^-$ production. It can therefore be concluded that piroxicam interferes in the mechanism by which the NADPH-oxidase is activated. For this reason piroxicam can potentially be very useful for investigations designed to elucidate the mechanism by which NADPH-oxidase is stimulated.

Overall it seems reasonable to believe that piroxicam, and possibly other NSAIDs, cause improvement in RA and osteoarthritis not

only by inhibition of prostaglandin synthesis but also by decrease of the $O_2^-$ production of PMN and macrophages, which results in the prevention of tissue destruction caused by oxygen free radicals.

ACKNOWLEDGEMENTS

We wish to thank Pfizer for estimation of the piroxicam concentrations, Ms. I. Simon for assistance by obtaining the samples, Mrs. E.M.W. van Zaal-de Korte for preparing the manuscript, Dr. R.N. Lemon for correction of the English, and the Rotterdam Rheumatism Foundation for their support.

REFERENCES

1. Halliwell B, Gutteridge JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1, 1984.
2. Lunec J, Halloran SP, White AG, Dormandy TL: Free-radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J Rheumatol 8:233, 1981.
3. Greenwald RA, Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum 23:455, 1980.
4. Blake DR, Hall ND, Bacon PA, Dieppe PA, Halliwell B, Gutteridge JMC: The importance of iron in rheumatoid disease. Lancet ii,1142, 1981.
5. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanisms: the production by leukocytes of superoxide, a potent bactericidal agent. J Clin Invest 52:741, 1973.
6. Freeman BA, Crapo JD: Biology of disease. Free radicals and tissue injury. Lab Invest 47:412, 1982.
7. Greenwald RA, Moy WW, Lazarus D: Degradation of cartilage proteoglycans and collagen by superoxide radical [abstract]. Arthritis Rheum 19:799, 1976.
8. Simchowitz L, Mehta J, Spilberg I: Chemotactic factor-induced generation of superoxide radicals by human neutrophils. Effect of metabolic inhibitors and anti-inflammatory drugs. Arthritis Rheum 22:755, 1979.

9. Harth M, Keown PA, Orange JF: Monocyte dependent excited oxygen radical generation in rheumatoid arthritis inhibition by gold sodium thiomalate. J Rheumatol 10:701, 1983.
10. Gay JC, Lukens JN, English DK: Differential inhibition of neutrophil superoxide generation by non-steroidal anti-inflammatory drugs. Inflammation 8:209, 1984.
11. Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol 231:232, 1971.
12. Edelson HS, Kaplan HB, Korchak HM, Smolen JE: Dissociation by piroxicam of degranulation and superoxide anion generation from decrements in chlortetracyclin fluorescence of activated human neutrophils. Biochem Biophys Res Commun 104:247, 1982.
13. Abramson S, Edelson H, Kaplan H, Given W, Weissmann G: The inactivation of the polymorphonuclear leukocyte by non-steroidal anti-inflammatory drugs. Inflammation 8:s103, 1984.
14. Kaplan HB, Edelson HS, Korchak HM, Given WP, Abramson S, Weissmann G: Effects of non-steroidal anti-inflammatory agents on human neutrophil functions in vitro and in vivo. Biochem Pharmacol 33:371, 1984.
15. Riedel KD, Laufen H: High-performance thin-layer chromatographic assay for the routine determinations of piroxicam in plasma, urine and tissue. J Chromatogr 276:243, 1983.
16. Weening RS, Roos D, Loos JA: Oxygen consumption of phagocytozing cells in human leukocyte and granulocyte preparations: a comparative study. J Lab Clin Med 83:570, 1974.
17. Tauber AI, Goetzl EJ: Structural and catalytic properties of the solubilized superoxide-generating activity of human polymorphonuclear leukocytes. Solubilization, stabilization in solution, and partial characterization. Biochemistry 18:5576, 1979.
18. Rister M, Bauermeister K: Superoxide-Dismutase und Superoxide-Radikal-Freizetsung bei juveliner rheumatoider Arthritis. Klin Wochenschr 60:561, 1982.
19. Chiu PL, Davis P, Wong K, Dasgupta M: Superoxide production in neutrophils of patients with rheumatoid arthritis and Felty syndrome. J Rheumatol 10:694, 1983.

20. Gale R, Bertouch JV, Bradley J, Roberts-Thomsom PJ: Direct activation of neutrophil chemiluminescence by rheumatoid sera and synovial fluid. Ann Rheum Dis 42:158, 1983.

21. Biemond P, Van Eijk HG, Swaak AJG, Koster JF: Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. J Clin Invest 73:1576, 1984.

22. Abramson S, Edelson HS, Kaplan HB, Ludewig R, Weissmann G: Inhibition of neutrophil activation by non-steroidal anti-inflammatory drugs. Am J Med 77:3, 1984.

23. Curnutte JT, Badwey JA, Robinson JM, Karnovsky MJ, Karnovsky ML: Studies on the mechanism of superoxide release from human neutrophils stimulated with arachidonate J Biol Chem 259:11851, 1984.

# CHAPTER 8

# GENERAL DISCUSSION

## PROTECTIVE FACTORS AGAINST OXYGEN FREE RADICALS IN RHEUMATOID ARTHRITIS

Synovial fluid from rheumatoid arthritis (RA) patients was examined for the amount of protective factors present (chapter 2). Superoxide dismutase, catalase and glutathione peroxidase were present in minor concentrations, unable to provide any protection. The transferrin concentration in synovial fluid is about 50% of the amount normally found in serum. This transferrin level is adequate to bind 4 times the amount of iron present in synovial fluid. The biological activity of transferrin is decreased, however. Gutteridge et al. (67) showed that transferrin in RA synovial fluid is unable to bind iron, possibly due to free radical attack. As a result transferrin, although present in considerable amounts is unable to prevent iron catalysed hydroxyl radical formation.

Ceruloplasmin was found in RA synovial fluid in a concentration two times higher than in controls. Blake et al. (68) showed that ceruloplasmin in RA synovial fluid has lost 60% of its activity, due to free radical attack. By using a lipid peroxidation test system we were able to show a protective effect of synovial fluid. Analysis revealed that ceruloplasmin was responsible for the inhibitory effect of synovial fluid on lipid peroxidation. The protection of ceruloplasmin is based on its enzymatic activity. In fact ceruloplasmin is a ferroxidase, converting ferrous ($Fe^{++}$) to ferric ($Fe^{+++}$) iron. This interferes with the reaction between $Fe^{++}$ and $H_2O_2$, blocking the hydroxyl radical formation.

It can be concluded that synovial fluid depends on ceruloplasmin for its protection against oxygen free radicals. Although RA patients are not deficient for this protein, it is doubtfull whether the activity of the protein causes sufficient protection. The presence of lipid peroxidation products in RA synovial fluid suggest it is insufficient (57, 59).

Glutathione is important in the protection against oxygen free radicals, partially because it is a scavenger, but more important it is needed for the activities of glutathione peroxidase and peroxidation inhibiting protein. Up to now only absolute amounts of GSH were measured (69, 70) and minor changes were found. The results of the

present study (chapter 3) show no difference in GSH concentration in erythrocytes of RA patients and controls. Additionally we found that during oxidative stress the regeneration of GSH is the same in RA and controls. Although measured in erythrocytes, the results indicate that it is unlikely that changes in intracellular GSH are involved in the etiology of RA.

## AVAILABILITY OF IRON ABLE TO CATALYSE THE FORMATION OF HYDROXYL RADICALS

Non-protein bound iron will catalyse the conversion of relative innocent oxygen free radicals like superoxide and hydrogen peroxide, which are produced by polymorphonuclear leucocytes (PMN), to very aggresive oxygen species like the hydroxyl radical. This potentiating effect stimulated to investigate the availability of free iron in RA. Only minimal quantities of iron are bound to low molecular weight chelators, enabling intracellular iron transport. Practically all the iron present in the body can be found in enzymes, haem-containing proteins, or in specific iron-binding proteins such as transferrin, ferritin or lactoferrin.

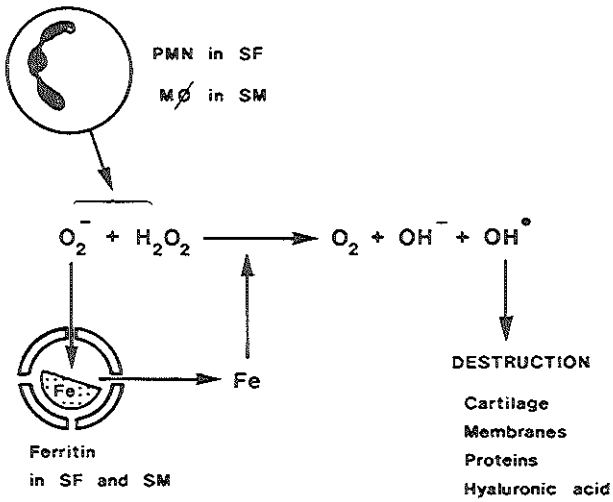



Fig.1: Hypothesis about the role of ferritin in the pathogenesis of rheumatoid arthritis. SF: synovial fluid, SM: synovial membrane, OH•: hydroxyl radical, MØ: macrophage

Iron mobilization from ferritin by superoxide

While nearly all iron is protein bound, the possibility of iron mobilization during inflammation was investigated (chapter 4, 6). It turned out that stimulated granulocytes were able to release iron from ferritin. Superoxide was shown to be responsible for the iron release, because superoxide dismutase blocked it. Solid potassium superoxide ($KO_2$) and superoxide produced by xanthine oxidase were also able to release iron from ferritin. Iron mobilization from transferrin could not be obtained by superoxide. This result has very important implications. During inflammation phagocytosing PMN and macrophages produce large amounts of superoxide. Iron is mobilized from ferritin by superoxide, and becomes available for catalysation of the hydroxyl radical formation, resulting in extensive tissue damage (figure 1). This mechanism is not only important in RA but in every situation in which superoxide is produced by PMN, or by other sources, in close association with adequate amounts of ferritin. In hemochromatosis for example this mechanism is very likely to occur, but also in many other inflammatory diseases.

The presence of ferritin and its iron saturation in the rheumatoid joint

The knowledge that ferritin is not a safe storage molecule for iron in relation to oxygen free radicals stimulated to study the amount of ferritin and especially its iron loading present in the rheumatoid joint (chapter 5). In synovial fluid ferritin is much increased compared to controls, and in contrast to what is suggested by Blake et al. (71), ferritin contains a considerable amount of iron. In synovial membrane iron bound to ferritin is three times increased in RA. These results clearly indicate that in RA patients iron bound to ferritin is present in the synovial fluid and the synovial membrane in amounts sufficient to provide iron for the formation of the hydoxyl radical.

In RA the saturation of ferritin in synovial fluid was only 50% of the saturation in the synovial membrane. This difference is interesting because it is very likely that synovial fluid ferritin originates from synovial membrane cells, and an equal saturation would be expected. The decreased saturation in synovial fluid can be ex-

plained by iron mobilization by superoxide from PMN, so this result supports our hypothesis.

## HYDROXYL RADICAL FORMATION IN ISCHEMIA

During ischemia xanthine oxidase is formed by the conversion of xanthine dehydrogenase. Xanthine oxidase is a well known source of superoxide and hydrogen peroxide. The present study (chapter 6) shows that xanthine oxidase will cause iron release from ferritin. About 70% is dependent on superoxide but a superoxide-independent mechanism of iron release by xanthine oxidase also exists. Iron mobilization from ferritin by xanthine oxidase is probably essential in the tissue damage in ischemia. Until now no source of non-protein bound iron was identified present during ischemia. With the new information the hypothesis about the role of oxygen free radicals in ischemic tissue destruction can be extended (figure 2): During ischemia xanthine oxidase and xanthine are formed. In the absence of oxygen, iron can be mobilized from ferritin by the superoxide-independent mechanism. The iron mobilization will increase in the presence of oxygen which is available due to partial ischemia, or after reperfusion. Superoxide production will occur in the presence of "free" iron, mobilized from ferritin, resulting in hydroxyl radical formation and tissue destruction. This mechanism can be inhibited at several levels. Allopurinol and superoxide dismutase have already been shown to be effective in myocardial ischemia *in vivo* (51). Iron chelators like desferrioxamin are not tested yet, but are very promising.

Xanthine oxidase might also be important in RA. Xanthine dehydrogenase is found in almost every organ in the body (72). During inflammation the conversion to xanthine oxidase is likely to occur due to the activity of proteases or due to relative ischemia (55). Xanthine oxidase will cause additional production of oxygen free radicals and iron mobilization from ferritin, which will increase tissue destruction in RA.

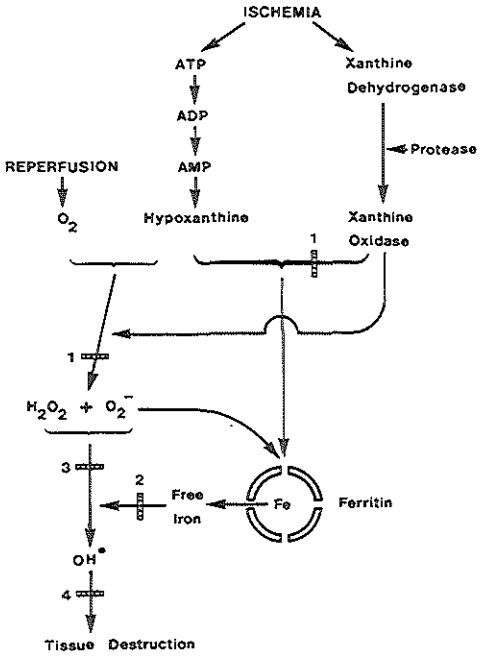



Fig.2: Xanthine oxidase and iron mobilized from ferritin in tissue destruction in ischemia. Inhibitors: 1 allopurinol ; 2 desferrioxamin: 3 SOD, catalase; 4 radical scavengers.

## INDICATION OF IRON ADMINISTRATION IN RHEUMATOID ARTHRITIS

In view of the danger of iron, related to the catalysation of the hydroxyl radical formation, iron should be handled as very dangerous. In my opinion iron suppletion is given much too often. It is not unusual to start iron treatment only on the basis of anemia, without any information about its origin. The deleterious effects of iron in RA are clearly illustrated by the fact that arthritis is frequently found in patients with hemochromatosis or hemophilia, diseases in which iron accumulates in the joint. More direct evidence is the observation that RA patients receiving intravenous iron, showed an exacerbation of their arthritis (73).

During active inflammation every RA patient is anemic. The serum iron is decreased due to the "anemia of inflammation". The most reli-

able parameter for real iron deficiency is the presence of iron in the bone-marrow. To prevent bone-marrow aspiration the serum ferritin concentration also can be used. However, interpretation of the ferritin concentration must be done very carefully. The inflammatory process itself causes an increase in serum ferritin, so special reference values have to be used in RA. Arbitrarily a ferritin serum concentration of 60 µg/l is chosen. Above this value nearly no patients are found without any stainable bone-marrow iron, and iron treatment is not indicated. If the ferritin serum concentration in RA patients is below 60 µg/ml, the bone marrow does not contain any iron in 86%, indicating real iron deficiency, and iron suppletion should be started (74, 75).

INHIBITION OF SUPEROXIDE PRODUCTION FROM GRANULOCYTES BY PIROXICAM

Non-steroidal anti-inflammatory drugs (NSAID) are inhibitors of cyclo-oxygenase as shown by Vane (76). For years this was accepted as the only therapeutic effect. It has to be kept in mind, however, that a considerable part of all prostaglandins deminish inflammation, and their production is also inhibited by NSAID. Previously NSAID have been shown to inhibit superoxide production by PMN *in vitro* (64, 66). The present results (chapter 7) show that treatment of patients suffering from RA or osteoarthritis, with piroxicam cause a decrease of $O_2^-$ production of their blood PMN after isolation. PMN isolated from synovial fluid were also inhibited in their superoxide production by piroxicam treatment (unpublished). So it is very likely that *in vitro* data are also valid *in vivo*. In addition information is obtained about the level of interaction between piroxicam and $O_2^-$ production by PMN. Piroxicam interferes with the activation of the NADPH-oxidase complex. It can be concluded that the beneficial effect of NSAID like piroxicam depends partially on a decrease in $O_2^-$ production by granulocytes.

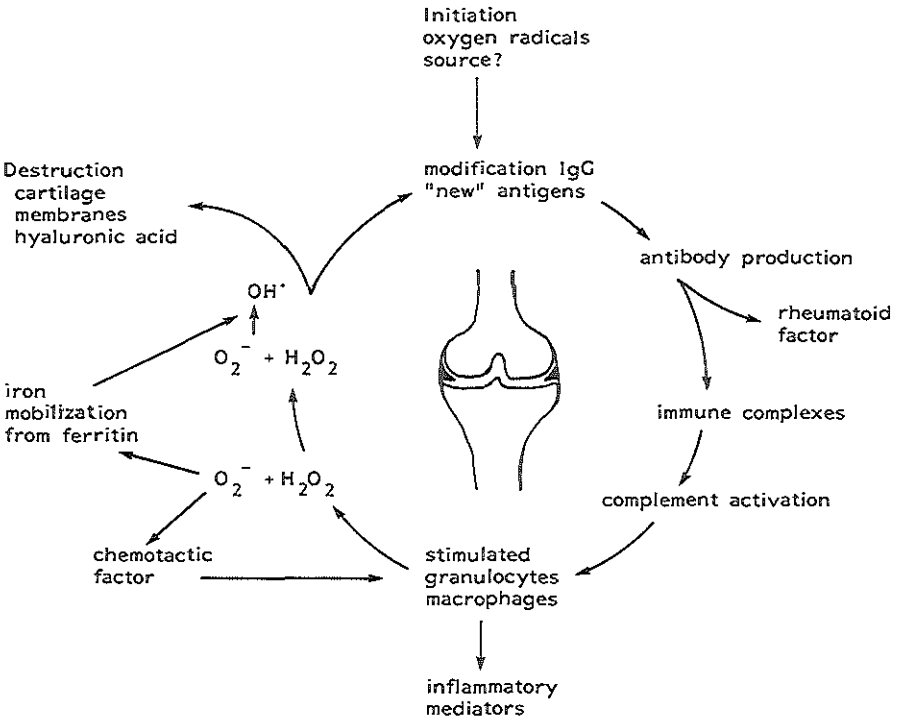



Fig. 3: Oxygen free radicals in the etiology of rheumatoid arthritis.

In the near future interesting results can be expected from research about the role of oxygen free radicals in RA.

The joint in RA contains high concentrations of ferritin loaded with iron, which can be mobilized by $O_2^-$. In view of the deleterious effects of iron in oxygen free radical tissue destruction, a new line of treatment of RA patients have to be considered: The selective removal of iron from the joint, by using iron chelators like desferrioxamin.

Many investigations strenghten the role of oxygen radicals in the pathogenesis of tissue damage in RA. However, recent results indicate that free radicals might also be involved in the etiology of RA. Human immunoglobulin is shown to be changed by oxygen free radical attack (77, 78). We were able to reproduce this result (unpublished). This result can be incorporated in a new hypothesis about the etiology of RA (figure 3). An initiating source of oxygen free radicals (still to be identified) modifies immunoglobin G leading to the exposition of new antigens. This can explain the formation of rheumatoid factors. Immune complexes will be formed, initiating an inflammatory reaction. Superoxide from PMN will release iron from ferritin, able to catalyse the formation of hydroxyl radicals, which will cause tissue damage but also will modify immunoglobulin G. This vicious circle can explain the chronicity of RA.

Of course many features still have to be resolved. For instance what is the initiating source of free radicals, and why are not all inflammatory processes leading to RA. These are important questions and the answers to them will certainly contribute to the treatment and eventually to the prevention of RA.

# CHAPTER 9

## SUMMARY

Evidence is increasing that oxygen free radicals are involved in the pathogenesis of rheumatoid arthritis (RA). Stimulated granulocytes macrophages produce large amounts of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$). Products typical for free radical damage are found in synovial fluid.

The present study shows that no enzymatic protection against oxygen free radicals is present in synovial fluid. Transferrin is found in synovial fluid, but other studies indicate that it has lost its capacity to bind iron, which is essential for its protection against free radical damage. Ceruloplasmin is shown to offer some protection of synovial fluid. Reduced glutathione (GSH) in the erythrocyte is not decreased in RA, and more importantly the capacity to regenerate GSH during oxidative stress is unimpaired. In comparison to controls there is no absolute deficiency in protective factors in RA, but the protection is insufficient to cope with the enourmous amount of radicals which are produced during active inflammation.

Iron is important in free radical damage because it catalyses the conversion of the rather innocent superoxide radical to the extremely reactive hydroxyl radical ($OH^\cdot$). Only free iron is able to stimulate $OH^\cdot$ formation. In the current thesis it is shown that $O_2^-$ is able to release iron from ferritin. Ferritin could be detected in both synovial fluid and synovial membrane in high concentrations, and contained a considerable amount of iron. These data suggest that oxygen free radical damage in RA is stimulated by the release of iron from ferritin by $O_2^-$ produced by granulocytes, resulting in the formation of the extremely toxic hydroxyl radical. This mechanism elucidates an important part of tissue destruction in RA.

Previous investigations indicate that non-steroidal anti-inflammatory drugs (NSAID) are able to inhibit $O_2^-$ production by granulocytes <u>in vitro</u>. Piroxicam treatment revealed that also <u>in vivo</u> inhibition of $O_2^-$ production can be achieved. Without knowing, for several decades RA is treated by inhibition of $O_2^-$ production, by the use of NSAID.

# CHAPTER 10

## POPULAIRE SAMENVATTING

Reuma is een vaak voorkomende ziekte waarbij er een langdurige ontsteking van gewrichten optreedt. De ernst van de ontsteking wisselt vaak in de loop van de tijd en leidt meestal tot beschadiging van het gewricht: kraakbeen verlies, verdikking van het gewrichtskapsel en botaantasting. Tevens gaat de kwaliteit van het gewrichtsvocht als smeermiddel verloren. Het gevolg is ruwe gewrichtsvlakken die over elkaar heen schuren. Zowel de actieve ontsteking als de verwoeste gewrichten veroorzaken de patient veel pijn.

De oorzaak van reuma is onbekend. De beschikbare kennis wijst er op dat een combinatie van factoren uiteindelijk reuma veroorzaakt. Het onderzoek dat in dit proefschrift is beschreven richt zich op een oorzakelijke factor waarvan nog weinig bekend is, namelijk de rol die giftige zuurstofprodukten spelen in het ontstaan van reuma.

## BESCHERMING TEGEN GIFTIGE ZUURSTOFPRODUKTEN

Tijdens de onsteking maken ontstekingscellen grote hoeveelheden giftige zuurstofprodukten, de zogenaamde zuurstof radicalen. Deze giftige zuurstofprodukten zijn in staat het gewricht ernstig te beschadigen. De eerste vraag was of in het gewrichtsvocht beschermende factoren aanwezig zijn om de gevormde giftige zuurstofprodukten onschadelijk te maken. Het bleek dat slechts een eiwit, het ceruloplasmine, in gewrichtsvocht enige bescherming biedt, echter deze bescherming is duidelijk onvoldoende.

## TOENAME VAN BESCHADIGING DOOR GIFTIGE ZUURSTOFPRODUKTEN ONDER INVLOED VAN IJZER

Het is bekend dat "vrij" ijzer minder giftige zuurstofprodukten omzet in extreem giftige soorten. Om dit te voorkomen is ijzer in het lichaam veilig opgeborgen in eiwitten zoals ferritine. In het beschreven onderzoek is aangetoond dat de ontstekingscellen een giftig zuurstofprodukt maken dat ijzer uit ferritine vrijmaakt. Het vrijgemaakte ijzer zal de vorming van secundaire, meer giftige zuurstofprodukten, doen toenemen. Het vrijmaken van ijzer uit ferritine wordt bevorderd doordat de hoeveelheid ijzer opgeslagen in ferritine groter is bij reuma patienten dan bij gezonde mensen.

Deze resultaten tonen een voorheen onbekend mechanisme van weefselbeschadiging in reuma aan.

## BESTRIJDING VAN HET ONGUNSTIGE EFFECT VAN IJZER

De ongunstige rol van ijzer tijdens een ontsteking heeft belangrijke gevolgen voor de behandeling van reuma:

1. Het voorschrijven van ijzer aan reuma patienten moet worden beperkt.
   Bloedarmoede komt erg vaak voor bij reumapatienten. Soms wordt dit veroorzaakt door echt ijzergebrek, echter meestal is er wel voldoende ijzer maar kan het niet goed worden gebruikt. Vaak wordt ijzer voorgeschreven aan reumapatienten, zonder dat een echt ijzertekort is aangetoond, onder het motto: "Baat het niet, dan schaadt het niet". Helaas schaadt het wel. Toename van de hoeveelheid ijzer zal de hoeveelheid zeer giftige zuurstofprodukten doen toenemen. Het is duidelijk dat ijzer alleen aan een reuma patient mag worden voorgeschreven als er een echt ijzer tekort is aangetoond.
2. De hoeveelheid ijzer in het gewricht moet worden verlaagd. De verkregen gegevens zijn een basis om een nieuwe, hopelijk meer effectieve behandeling van reuma te testen. Door toediening van medicijnen die ijzer afvoeren uit het gewricht kan de ontsteking waarschijnlijk worden geremd.

## DE INVLOED VAN BEKENDE GENEESMIDDELEN TEGEN REUMA OP GIFTIGE ZUURSTOFPRODUKTEN

Aspirine in hoge dosering en een groot aantal sterk verwante medicijnen remmen de ontsteking in reumapatienten. Een van deze geneesmiddelen, namelijk piroxicam is getest en bleek de aanmaak van giftige zuurstofprodukten te remmen. Dit resultaat geeft meer inzicht in hoe deze middelen werken bij reuma.

CONCLUSIE

De gegevens uit dit proefschrift versterken de argumenten dat giftige zuurstofprodukten een rol spelen in het onstaan van reuma. Hopelijk zal de behandeling van reumapatienten verbeteren door:

- Het beperken van het voorschrijven van ijzer.
- Het gebruik van nieuwe geneesmiddelen die de hoeveelheid ijzer in het gewricht doen afnemen.
- Het gebruik van middelen die tegen giftige zuurstofprodukten beschermen.

# GENERAL REFERENCES

1. Ropes MW, Bennett GA, Cobb S., Jacox R, Jessar RA: 1958 revision of diagnostic criteria for rheumatoid arthritis. Bull Rheum Dis 9:175, 1958.
2. Kelley WN, Harris ED, Ruddy S, Sledge CB (ed): Textbook of rheumatology. Second edition, WB Saunders Company, Philadelphia, USA, 879, 1985.
3. Wolfe AM: The epidemiology of rheumatoid arthritis: A review. Bull Rheum Dis 19:518, 1968.
4. McMichael AJ, Sasazubi T, McDevitt HO, Payne RO: Increased frequency of HLA-3C and HLA-Dw4 in rheumatoid arthritis. Arthritis Rheum 20:1037, 1977.
5. Stasny P: Association of the B-cell alloantigen DR4 with rheumatoid arthritis. N Engl J Med 298:869, 1978.
6. Solinger AM, Bhatnagar R, Stabo JD: Cellular, molecular and genetic characteristics of T cell reactivity to collagen in man. Proc Natl Acad Sci (USA) 78:3877, 1981.
7. Mansson I, Olhagen B: Fecal clostridium perfringens and rheumatoid arthititis. J Infect Dis 130:444, 1974.
8. Stewart SM, Alexander WRM, Duthie JJR: Isolation of diphtheroid bacilli from synovial membrane and fluid in rheumatoid arthritis. Ann Rheum Dis 28:477, 1969.
9. Ross RF: Pathogenicity of swine mycoplasmas. Ann N Y Acad Sci 225:347, 1973.
10. Alspaugh MA, Tan EM: Serum antibody in rheumatoid arthritis reactive with a cell-associated antigen: Demonstration by precipitation and immunofluoresence. Arthritis Rheum 19:711, 1976.
11. Hasler F, Bluestien HG, Zvailer NJ, Epstein LB: Analysis of the defects responsible for the impaired regulation of Epstein-Barr virus-induced B cell proliferation by rheumatoid arthritis lymphocytes. J Exp Med 157:173, 1983.
12. Andriopoulos NA, Mostecky J, Wright GP et al.: Characterization of antibodies to the native human collagens and to their component α-chains in the sera and the joint fluids of patients with rheumatoid arthritis. Immunochemistry 13:709, 1976.
13. Trentham DE, Townes AS, Kang AH: Autoimmunity to type II collagen: An experimental model of arthritis. J Exp Med 146:857, 1977.

14. Cats A, Hazevoet HM: Significance of positive tests for rheumatoid factor in the prognosis of rheumatoid arthritis. Ann Rheum Dis 29:254, 1970.
15. Smiley JD, Sachs C, Ziff M: In vitro synthesis of immunoglobulin by rheumatoid synovial membrane. J Clin Invest 47:624, 1968.
16. Bland JH, Clark L: Rheumatoid factor in serum and joint fluid. Ann Int Med 58:829, 1963.
17. Albert ED, Gotze D: The major histocompatibility system in man. In: The Major histocompatibility System in Man and Animals. Gotze D (ed) Berlin, Springer-Verlag, 1977.
18. Lee GR: The anemia of chronic disease. Semin Hematol 20:61, 1983.
19. Niedermeier W, Griggs JH: Trace metal composition of synovial fluid and blood serum of patients with rheumatoid arthritis. J Chronic Dis 23:528, 1971.
20. Freeman BA, Crapo JD: Biology of disease. Free radicals and tissue injury. Lab Invest 47:412, 1982.
21. Turrens JF, Boveris A: Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 191:421, 1980.
22. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanism. The production by leucocytes of superoxide, a potent bactericidal agent. J Clin Invest 52:741, 1973.
23. Mason RP: Free-radical intermediates in the metabolism of toxic chemicals. In: Free radicals in Biology, vol 5. Pryor WA (ed) Acad Press, New York, 161, 1982.
24. McGinnes JE, Proctor PH, Demopoulos HB, Hokanson JA, Van NT: An in vivo enzymatic probe for superoxide and peroxide production by chemotherapeutic agents. In: Pathology of Oxygen, Autor P (ed), Acad Press, New York, 192, 1982.
25. Haber F, Weiss J: The catalytic decomposition of hydrogen peroxide by iron salts. Proc Roy Soc London A147:332, 1934.
26. Aust SD, Svingen BA: The role of iron in enzymatic lipid peroxidation. In: Free radicals in Biology, vol 5. Pryor WA (ed) Acad Press, New York, 1, 1982.
27. Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. FEBS Lett 92:321, 1978.

28. Winterbourn CC: Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate and superoxide. Biochem J 198:125, 1981.
29. Gutteridge JMC, Rowley DA, Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of "free" iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199:263, 1981.
30. Stocks J, Gutteridge JMC, Sharp RJ, Dormandy TL: The inhibition of lipid autoxidation by human serum and its relation to serum proteins and α-tocopherol. Clin Sci Mol Med 47:223, 1974.
31. Gutteridge JMC, Paterson SK, Segal AW, Halliwell B: Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J 199:259, 1981.
32. McCord JM, Day ED: Superoxide-dependent production of hydroxyl radical catalysed by iron-EDTA complex. FEBS Lett 86:139, 1978.
33. Ambruso DR, Johnston RB: Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fraction and an enzymatic generating system. J Clin Invest 67:352, 1981.
34. Bannister JV, Bannister WH, Hill HAO, Thornalley PJ: Enhanced production of hydroxyl radicals by the xanthine-xanthine oxidase reacation in the presence of lactoferrin. Biochim Biophys Acta 715:116, 1982.
35. Motohashi N, Mori I: Superoxide-dependent formation of hydroxyl radical catalysed by transferrin. FEBS Lett 157:197, 1983.
36. Baldwin DA, Jenny ER, Aisen P: The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J Biol Chem 259:13391, 1984.
37. Wills ED: Mechanisms of lipid peroxide formation in animal tissues. Biochem J 99:667, 1966.
38. Gutteridge JMC, Halliwell B, Treffry A, Harrison PM, Blake D: Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem J 209:557, 1983.
39. Carlin G, Djursäter R: Xanthine oxidase induced depolymerization of hyaluronic acid in the presence of ferritin. FEBS Lett 177:27, 1984.
40. Lawrence JH, Loomis WF, Tobias CA, Turpin FH: Preliminary observations on the narcotic effect of xenon with a review of values for solubilities of gases in water and oils. J Physiol 105:197, 1946.

41. Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C: Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. Biochim Biophys Acta 710:197, 1982.
42. McCord JM, Roy RS: The pathophysiology of superoxide: Roles in inflammation and ischemia. Can J Physiol Pharmacol 60:1346, 1982.
43. Fantone JC, Ward PA: Role of oxygen-derived free radicals and metabolites in leucocyte-dependent inflammatory reactions. Am J Pathol 107:395, 1982.
44. Halliwell B: Production of superoxide, hydrogen peroxide and hydroxyl radicals by phagocytic cells; a cause of chronic inflammatory disease? Cell Biol Int Rep 6:529, 1982.
45. Petkau A, Chelak WS, Pleskach SD: Protection by superoxide dismutase of white blood cells in x-irradiated mice. Life Sci 22:867, 1978.
46. Edsmyr F: Superoxide dismutase efficacy in ameliorating side effects of radiation therapy: double blind placebo controlled trial in patients with bladder and prostate tumors. In: Pathology of Oxygen. Autor AP (ed) Acad Press, New York, 315, 1982.
47. McLennan G, Autor AP: Effect of intraperitoneally administered superoxide dismuate on pulmonary damage resulting from hyperoxia. In: Pathology of Oxygen. Autor PA (ed) Acad Press, New York, 85, 1982.
48. Tate RM, Repine JE: Phagocytes, oxygen radicals and lung injury. In: Free radicals in Biology, vol 6. Pryor WA (ed) Acad Press, New York, 199, 1984.
49. Batelli MG, Corte ED, Stirpe F: Xanthine oxidase type D (dehydrogenase) in the intestine and other organs of the rat. Biochem J 126:747, 1972.
50. Granger DM, Rutili G, McCord JM: Superoxide radicals in feline intestinal ischemia. Gastroenterol 81:22, 1981.
51. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM: Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol 17:145, 1985.

52. McCord JM: Mechanisms of disease: Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312:159, 1985.
53. Crowell JW, Jones CE, Smith EE: Effect of allopurinol on hemorrhagic shock. Am J Phsyiol 216:744, 1969.
54. Dewall RA, Vasco KA, Stanley EL, Kezdi P: Responses of the ischemic myocardium to allopurinol. Am Heart J 82:362, 1971.
55. Lund-Olesen K: Oxygen tension in synovial fluids. Arthritis Rheum 13:769, 1970.
56. Muus P, Bonta IL, Den Oudsten SA: Plasma levels of malondialdehyde, a product of cyclo-oxygenase-dependent and independent lipid peroxidation in rheumatoid arthritis: a correlation with disease activity. Prostaglandins Med 2:63, 1979.
57. Rowley D, Gutteridge JMC, Blake D, Farr M, Halliwell B: Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid arthritis patients. Clin Sci 66:691, 1984.
58. Lunec J, Dormandy TL: Fluorescent lipid peroxidation products in synovial fluid. Clin Sci 56:53, 1979.
59. Lunec J, Halloran SP, White AG, Dormandy TL: Free-radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J Rheumatol 8:233, 1981.
60. Greenwald RA, Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum. 23:455, 1980.
61. Menander-Huber K: Double blind controlled trials in man with bovine copper zinc superoxide dismutase (orgotein). In: Biological and clinical aspects of superoxide and superoxide dismutase. Bannister WH, Bannister JV (eds) Elsevier, Amsterdam, 408, 1980.
62. Goebel KM, Storck U, Neurath F: Intrasynovial orgotein therapy in rheumatoid arthritis. Lancet i:1015, 1981.
63. Younes M, Weser U: Superoxide dismutase activity of copper-penicillamine: possible involvement of Cu(I) stabilized sulphur radical. Biochem Biophys Res Commun 78:1247, 1977.
64. Simchowitz L, Mehta J, Spilberg I: Chemotactic factor-induced generation of superoxide radicals by human neutrophils. Effect of metabolic inhibitors and anti-inflammatory drugs. Arthritis Rheum 22:755, 1979.

65. Harth M, Keown PA, Orange JF: Monocyte dependent excited oxygen radical generation in rheumatoid arthritis: Inhibition by gold sodium thiomalate. J Rheumatol 10:701, 1983.

66. Abramson S, Edelson H, Kaplan H, Given W, Weissmann G: The inactivation of the polymorphonuclear leucocyte by non-steroidal anti-inflammatory drugs. Inflammation 8:s103, 1984.

67. Gutteridge JMC, Halliwell B, Rowley DA: Catalytic iron complexes in biological material: A potential for oxygen radical damage. Life Chem Rep S2:15, 1984.

68. Blake DR, Blann A, Bacon PA, Farr M, Gutteridge JMC, Halliwell B: Ferroxidase and ascorbate oxidase activities of caeruloplasmin in synovial fluid from rheumatoid patients. Clin Sci 64:551, 1983.

69. Munthe E, Guldal, G, Jellum E: Increased intracellular glutathione during penicillamine treatment for rheumatoid arthritis. Lancet ii:1126, 1979.

70. Munthe E, Kass, E, Jellum E: D-Penicillamin-induced increase in intracellular glutathione correlating to clinical response in rheumatoid arthritis. J Rheumatol 8:14, 1981.

71. Blake DR, Bacon PA, Eastham EJ, Brigham K: Synovial fluid ferritin in rheumatoid arthritis. Brit Med J. 281:715, 1980.

72. Krenitsky TA, Tuttle JV, Cattau EL, Wang P: A comparison of the distributon and electon acceptor specificities of xanthine oxidase and aldehyde oxidase. Comp Biochem Physiol 49B:687, 1974.

73. Blake DR, Lunec J, Ahern M, Ring EFJ, Bradfield J, Gutteridge JMC: Effect of intravenous iron dextran on rheumatoid synovitis. Ann Rheum Dis 44:183, 1985.

74. Smith RJ, Davis P, Thomson ABR, Wadsworth LD, Fackre P: Serum ferritin levels in the anemia of rheumatoid arthritis. J Rheumatol 4:389, 1977.

75. Mørk Hansen T, Hansen NE, Birgens HS, Hølund B, Lorenzen I: Serum ferritin and the assessment of iron deficiency in rheumatoid arthritis. Scand J Rheumatol 12:353, 1983.

76. Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs. Nature New Biol 231:232, 1971.

77. Wickens DG, Norden AG, Lunec J, Dormandy TL: Fluorescence changes in human gamma globulin induced by free radical activity. Biochem Biophys Acta 742:607, 1983.

78. Lunec J, Blake DR, McClearly SJ, Brailsford S, Bacon PA: Self-perpetuating mechanisms of immunoglobulin G aggregation in rheumatoid inflammatory. J Clin Invest 76:2084, 1985.

OVERIGE PUBLICATIES

1. Biemond P, Swaak AJG, Koster JF: Pathologie door zuurstof. Ned Tijdschr Geneeskd 128:2230, 1984
2. Koster JF, Biemond P, Montfoort A, Stam H: The involvement of free radicals in pathological conditions. Life Chem Reports 3:323, 1986
3. Biemond P, Swaak AJG, Penders JMA, Beindorff CM, Koster JF: In vivo inhibition of superoxide production of granulocytes by the antirheumatic drug piroxicam. An interference with the activation of NADPH-oxidase. In: Superoxide and superoxide dismutase in chemistry, biology and medicine. Rotilio G (ed) Amsterdam, Elsevier Science Publishers, 541, 1986

## CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 24 mei 1956 te Zevenhuizen. Na het behalen van het Atheneum B diploma aan het Christelijk Lyceum te Gouda werd in 1974 met de studie Geneeskunde begonnen aan de Erasmus Universiteit Rotterdam. Tijdens de studie is als keuze-praktikant en aansluitend als student-assistent onderzoek gedaan naar de rol van trypsine in de resorptie van vitamine $B_{12}$ op de afdeling Hematologie (hoofd: Prof. Dr. J. Abels). In 1980 werd het artsexamen afgelegd. Het eerste half jaar van 1981 was hij werkzaam als arts-assistent gynaecologie en obstetrie in het St. Clara Ziekenhuis te Rotterdam (hoofd: Dr. J.M. Versteeg). Het resterende deel van het jaar werd als arts-assistent chirurgie gewerkt in het Bergweg Ziekenhuis te Rotterdam (hoofd: Dr J.W. Merkelbach). Vanaf begin 1982 werd gedurende twee jaar full-time aan het beschreven onderzoek gewerkt of de afdeling Biochemie I van de Medische Faculteit van de Erasmus Universiteit Rotterdam onder leiding van Prof. Dr. J.F. Koster en Dr. A.J.G. Swaak. Sinds 1 maart 1984 is hij in opleiding tot internist op de afdeling Inwendige Geneeskunde III van het Academisch Ziekenhuis Rotterdam/ Dijkzigt (opleider: Prof. Dr. J.C. Birkenhäger) in welke periode dit proefschrift werd afgerond.