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REVIEW

Iron in joint inflammation

Alya J Dabbagh, Charles W Trenam, Chris J Morris, David R Blake

The Inflammation Research Group, The London Hospital Medical College, 25-29 Ashfield Street, London E1 2AD, United Kingdom
A J Dabbagh
C W Trenam
C J Morris
D R Blake

Correspondence to: Dr Morris.

Iron and joint inflammation was first associated by Hochstatter in his description in 1674 of an arthritis associated with excessive bleeding,[1] effectively a description of the synovitis associated with haemophilia. Haemophilic arthropathy is characterised by a florid and proliferative synovitis and associated with erosive bone damage and cartilage destruction.[2] Bleeding into an otherwise normal joint is clearly associated with damage. In patients with an abnormal joint, for instance rheumatoid synovitis, iron may also play a part in perpetuating inflammatory damage.

Even though rheumatoid arthritis (RA) mainly affects peripheral synovial joints, it is also a systemic disease, being accompanied by anaemia, weight loss, and an increase in erythrocyte sedimentation rate. Vascular, cardiac, and pulmonary lesions are also produced. All rheumatoid patients with persistent inflammation develop the anaemia of chronic disease. This is a mild anaemia associated with chronic inflammatory conditions. It is characterised by a disturbance in iron metabolism, which results in hypoferraemia (low serum iron and haemoglobin concentrations), despite iron stores that range from adequate to raised.

Other features of the anaemia of chronic disease include a decrease in both plasma total iron binding capacity and transferrin saturation with iron. There is, however, a marked increase in serum ferritin, and in RA serum ferritin levels may reach 250% of initial values.[3 4]

The most widely accepted explanation for the low serum iron appears to be that iron deposited in the reticuloendothelial (RE) cells is not properly released to transferrin in the circulation—that is, it is poorly re-used.[5]

The RE system has a central role in iron metabolism, processing haemoglobin from senescent erythrocytes.[6] Under normal circumstances the RE system provides most of the iron required for erythropoiesis, and iron storage and release by the RE system are in equilibrium.

After a lag period due to haem catabolism the RE cells release iron into the plasma by a two phase process. The first is an early phase (immediate release from haem catabolism), which is completed within a few hours, and the second is a late phase corresponding to iron release from RE stores.[7]

Lee proposed two mechanisms for the impaired RE iron release during inflammation. The first is the liberation of the iron binding protein lactoferrin from the specific granules of leucocytes. Lactoferrin at low pH (<7·0) removes iron from the extracellular transport protein transferrin. The lactoferrin then returns the iron to the macrophage where it is stored as ferritin. Lactoferrin does not transfer iron to erythropoietic cells.[8] The second mechanism was first proposed by Konijn and Hershko who suggested that the hypoferraemia of inflammation results from an increase in intracellular synthesis of the iron storage protein apoferritin. Serum apoferritin behaves as an acute phase reactant and in acute inflammatory states it tends to parallel changes in another acute phase protein, haptoglobin. The apoferritin provides a storage depot for incoming iron delivered through lactoferrin or from effete erythrocytes. Stored iron is less available to the plasma iron transport system than iron recently derived from destroyed erythrocytes. Therefore, it would be expected that a diversion of iron into stores would result in hypoferraemia and an iron supply insufficient for erythropoiesis.[9]

Recently, Fillet and coworkers characterised RE iron kinetics in patients with inflammatory disease and in normal controls. As compared with normal subjects, the patients with inflammation had significantly lower serum iron concentrations and higher plasma ferritin concentrations. Early release of radiolabelled iron from the RE cells was considerably reduced and the late release markedly increased. There was an appreciable negative correlation of the percentage of early release with plasma ferritin, but no correlation was found between the percentage of early release and biological markers of inflammation (erythrocyte sedimentation rate, fibrinogen, or α_2 globulin). This showed that early release was decreased because RE stores were increased and not because of the intensity of inflammation itself.[10] Ferritin production is enhanced in the inflammatory RE cells,[9] and this precedes the decrease in serum iron concentrations. Thus Fillet *et al* (1989) have supported the hypothesis of increased ferritin synthesis as a mechanism for the reduction in iron output from RE cells.[10]

These changes are the result of the production of inflammatory mediators from a variety of different cell types. The increased synthesis of the acute phase proteins is driven by interleukin 1,[11] interleukin 6,[12] and tumour necrosis factor.[13] Specific acute phase proteins are regulated differently by the inflammatory mediators interleukin 1, interleukin 6, and tumour necrosis factor.[14]

Ferritin

The iron storage protein ferritin may be glycosylated within hepatocytes and secreted,

behaving as an acute phase protein in inflammation.[15] As well as increasing the synthesis of ferritin, interleukin 1 contributes towards the hypoferraemia by increasing lactoferrin production from the specific granules of neutrophils.[16] During inflammation lactoferrin competes with transferrin for iron (particularly at the low pH existing in inflammatory sites),[17] and it does not transfer iron to erythropoietic cells,[18] only to macrophages.[19] In 1989 Brock and Alvarez-Hernandez showed that tumour necrosis factor has a greater effect than interleukin 1 on the changes in iron metabolism associated with inflammation.[20] Tumour necrosis factor was shown to alter the ability of macrophages to take up, process, and release iron.

There are a strikingly large number of ferritin molecules in the rheumatoid synovial lining cells. Muirden was the first to suggest that the large amounts of iron sequestered in the rheumatoid synovial membrane may contribute towards the anaemia of the disease. The author found large amounts of iron loaded ferritin molecules, particularly in type A synovial lining cells. The ferritin was scattered throughout the cell cytoplasm but was often concentrated in lysosomes. All the synovial biopsy specimens which contained iron were removed from patients who were either severely or moderately anaemic.[21] Previous work by Muirden showed that the synovial lining cells of rabbits could take up ferritin,[22] and this was also shown to be true for iron dextran[23] when injected intra-articularly into the rabbit knee joint. Muirden concluded that the synovial intimal cells play an active part in the absorption of ferritin from the synovial cavity and that A cells played the major part in this uptake.[22] Ball *et al* detected a mild inflammatory reaction in the synovial membrane with some synovial proliferation five to 18 hours after the iron dextran injection. The iron was in the forms of ferritin and haemosiderin and persisted for three months after the injection.[23]

Muirden and coworkers showed that synovial cells in culture can ingest haemoglobin prepared from haemolysed red cells, and the subsequent appearance of ferritin in these cells implied that they were able to synthesise apoferritin. The authors suggested that both the synthesis of ferritin and the breakdown of haemoglobin take place within the same lysosome and that iron from lysed erythrocytes is likely to be an important source of the iron deposits in the rheumatoid synovia.[24] Muirden and Senator performed a light microscopic study of synovia from 23 rheumatoid patients and synovia from patients with other joint diseases. They showed that iron deposits are a constant feature of the pathology of RA. Prussian blue (Perls) positive staining, indicating the presence of ferric iron, was seen in all but one of the 27 synovial biopsy specimens. The exception, material processed for electron microscopy, showed ferritin granules in some of the surface cells. Haemosiderin granules were seen in 15 of the 27 biopsy specimens. The authors suggested that iron deposits in RA arise from continued oozing of blood from the vascular granulation tissue into the synovial cavity.[25] In highly inflamed rheumatoid joints, simple weight bearing or the stress of motion may compress the hyperplastic villi and synovial folds, leading to bleeding. These large deposits of iron may have a considerable contributory role in the anaemia and pathogenesis of this disease.

The role of iron in RA

Muirden provided further evidence for the link between synovial iron and the pathogenesis of RA.[26] In 28 patients there was a significant relation between the presence of anaemia due to RA and histological estimate of the extent of iron deposits. There was also a relation between the duration of the disease, the grade of radiographic change, and the extent of iron deposition.[26] An isotope kinetic method with ^{59}Fe labelled plasma transferrin was used to study the rate and mechanism of iron deposition in the synovial membrane in 13 rheumatoid knees.[27] Iron accumulation occurred only after incorporation of labelled iron into circulating erythrocytes. This led to the conclusion that intermittent intra-articular haemorrhages were the source of iron deposits in the rheumatoid synovia. This hypothesis has been tested using the rat allergic air pouch model, which produces a similar membrane structure to that of human synovium. The addition of autologous whole blood to this naturally remitting allergic model prolongs a low grade inflammatory state as in RA, where microbleeding occurs. The proinflammatory factor in these studies was present in erythrocytes and would appear to be haem iron,[28] rather than breakdown products of haemoglobin such as haemin, which depresses ferritin synthesis in vitro.[29] Macrophage ferritin synthesis was much enhanced in this model.[30]

The amount of ferritin within human rheumatoid synovial macrophages was shown to be associated significantly with the activity of early rheumatoid disease at the time of biopsy. In contrast, deposition of Perls' positive haemosiderin was associated with persistence of the disease.[30] The authors suggested that ferritin production may fail in a population of synovial macrophages, and that iron derived from effete erythrocytes is deposited in such a fashion as to induce the synthesis and release of collagenase and prostaglandin E_2 with consequent joint damage. Alternatively, the ferric iron may be reduced to the ferrous form and promote the formation of toxic free radical species.

Blake *et al*, expanding on this theme, suggested two possible mechanisms for the damage caused by the accumulation of iron in the rheumatoid synovial membrane.[31] Firstly, they suggested that iron catalyses oxidative radical reactions which lead to the formation of the hydroxyl radical ($^{\bullet}OH$) and subsequent lipid peroxidation. Hydroxyl radicals and lipid peroxidation cause extensive disruption of cellular organelle membranes and promote inflammatory tissue damage. Polyunsaturated fatty acids in plasma low density lipoproteins can be oxidised by iron, endothelial cells, and macrophages. This oxidised low density lipoprotein has chemotactic properties for monocytes and is cytotoxic to endothelial cells and smooth

muscle fibres. This group has recently shown the presence of both intracellular and extracellular staining for oxidised low density lipoprotein in the rheumatoid synovium. Intracellular staining was confined to foamy macrophages. Type A synoviocytes did not form foam cells but did show a surface staining pattern.[32] Secondly, the infiltration of the rheumatoid synovium by chronic inflammatory cells may be due to a tendency for these cells, which have receptors for iron binding proteins, to migrate towards deposits of iron.[33]

Iron was seen in 25% of the synovial cells from patients with RA.[34] The iron was usually deposited in siderosomes in type B cells, which were the predominant cell type, and no tissue damage was observed in the vicinity of these iron rich siderosomes. This is in contrast with the findings in type A cells, where siderosomes were associated with considerable cytoplasmic damage. The authors suggested that the apparent dominance of the type B cells may be due to type A cell damage and death, reflecting the capacity for free radical production in these macrophage-like cells in response to iron. Alternatively, there may be a failure of type A cells to produce an apoferritin response with the subsequent precipitation of intracellular proteins by iron. The authors also put forward the idea that the synovial cells may change their function, with a transition from A to B cell morphology as a result of inflammation.[35] This second hypothesis would explain the apparent correlation between the amount of iron in the synovia and the extent of erosive damage,[25] as it is the synovial B cells that have the capacity to cause local bone resorption through the generation of prostaglandin E_2. Another pertinent observation is that rheumatoid synovial cells are more sensitive to oxidative stress than normal cells, as shown by Rogers *et al*.[36] They showed that mild stress induced by free radicals (and also heat shock) readily caused a collapse of the intermediary filament, vimentin, from a transcytoplasmic to a perinuclear position.[36]

Low molecular weight iron chelates which are capable of catalysing $^{\bullet}OH$ formation and lipid peroxidation have been detected in rheumatoid synovial fluid[37] using the bleomycin method.[38] The concentration of this bleomycin-detectable iron correlated with the thiobarbituric acid reactive material in the synovial fluid and with indices of disease activity (knee score, leucocyte count, and synovial fluid C reactive protein). Recently, Grootveld *et al* (unpublished data), using Hahn spin echo and single pulse proton (^1H) nuclear magnetic resonance (NMR) spectroscopy combined with the use of powerful iron chelators have 'speciated', and confirmed the presence of these catalytic, non-transferrin bound iron, low molecular weight complexes in fresh synovial fluid samples; they characterised them as predominantly complexes with endogenous citrate, with the possible involvement of acetate and formate. These are in a form that can stimulate the generation of $^{\bullet}OH$ from the superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) via the Harber–Weiss reaction, by oxidant mediated mobilisation from ferritin and haemoglobin, especially in the acidotic environment of inflamed joints (Grootveld M C *et al*, unpublished data).

In rheumatoid synovial fluid the concentration of ferritin is considerably higher than in the serum.[39] This ferritin, however, contains very little iron.[15] Apoferritin alone has the ability to stimulate $O_2^{\bullet-}$ production from neutrophils, which are abundant in the rheumatoid synovial fluid. The $O_2^{\bullet-}$ can mobilise iron from ferritin,[40] which may explain the low iron loading of synovial fluid ferritin.

Further work by Blake and Bacon has shown a significant association between synovial fluid ferritin concentration, synovial immune complexes, and other indices of inflammatory activity in patients with RA. They suggested that this association may be due to a direct toxic effect of excess iron within the synovial RE cell, reducing its ability to clear immune complexes.[41]

Recently, Ahmadzadeh and coworkers[42] presented evidence supporting the findings of Blake and Bacon.[41 43] The authors measured the iron binding proteins, free and bound iron in the synovial fluid of 30 rheumatoid patients and compared them with those of patients with osteoarthritis. They demonstrated a significant increase in synovial fluid ferritin and lactoferrin but not transferrin. Despite a significant increase in bleomycin detectable iron, there was a decrease in the saturation index of synovial fluid ferritin and transferrin. Free or bound iron in synovial fluid correlated with indices of inflammatory activity such as rheumatoid factor and immune complex levels in the synovial fluid of patients with RA. These data suggest that iron participates in the local inflammatory process and has an important role in the pathogenesis of articular damage in patients with RA.[42 43]

Further evidence for the damaging effects of iron in RA is provided by the exacerbation of synovitis seen when iron is given to rheumatoid patients. In addition to the anaemia of chronic disease, some rheumatoid patients develop true iron deficiency anaemia. Figures of up to 75% of patients with RA have been quoted.[44] In these iron deficient patients there is a clinical need to provide iron in a safe and non-toxic form. Oral iron supplements in the form of ferrous sulphate produce a flare of the peripheral synovitis within 48 hours of ingestion.[44] This was shown by both clinical and laboratory indices. A large number of patients with RA receive multiple drug treatment (including penicillamine, which binds iron) and are intolerant of oral iron supplements. Intramuscular preparations of iron are difficult to administer owing to poor muscle bulk and local tenderness. In this group a total dose infusion of iron dextran is usually given. Reddy and Lewis (1969) reported that five of seven rheumatoid patients treated with intravenous iron dextran showed a flare in their arthritis. In each instance the flare took place within 24 hours of iron dextran administration, but only in those joints already affected by arthritis. The joints demonstrated increased swelling, heat, and pain.[45] We have attempted to mimic the clinical situation in which anaemic rheumatoid patients are given iron supplements in the presence of clinical joint symptoms by using the adjuvant arthritis

model in rats and giving iron in the presence of, rather than before, clinical manifestations of joint symptoms. Iron produced an exacerbation of joint inflammation between three and five days after injection.[46] This is similar to the findings of Lloyd and Williams, who showed that nine of 10 patients showed an exacerbation of arthralgia for up to seven days when given total dose infusion of iron dextran. The joint symptoms were accompanied by a feeling of general malaise, low grade fever, and a definite rise in erythrocyte sedimentation rate.[47]

Two studies by Blake's group[48 49] have provided clear evidence that iron catalysed reactions are the causative factors in the exacerbation of synovial inflammation consequent on total dose infusion of iron dextran. In the first study the authors showed that the exacerbation corresponded with saturation of serum iron binding capacity. The levels of immune complexes were unaltered, implying a normal RE cell function and hence ruling out the dextran as a causative factor. In one patient an increase in lipid peroxidation products (as measured by thiobarbituric acid reactive material and conjugated dienes) in the synovial fluid corresponded with the exacerbation of synovitis. The authors confirmed this by an in vitro study which showed that it was the iron component of the iron dextran complex which stimulated lipid peroxidation.

The second study showed that exacerbation of rheumatoid synovitis produced by total dose infusion of iron dextran corresponded with saturation of serum and synovial fluid iron binding capacity giving rise to low molecular weight iron chelates which can cause oxidative damage. Simultaneously, lipid peroxidation and the concentration of dehydroascorbate increased in both serum and synovial fluid. Hepatic function was transiently disturbed seven days after the infusion, implying hepatic oxidant stress within the iron loaded liver. A relatively new approach to the treatment of RA is the removal of excess iron within the joint. This manoeuvre has been successful in animal models, the iron chelator desferrioxamine reducing the incidence and severity of inflammation and associated soft tissue swelling and bone erosion in rat adjuvant polyarthritis.[50] In patients, however, desferrioxamine has been associated with cerebral and ocular toxicity[51 52] and is not orally active. A new orally active family of chelators, the hydroxypyridinones, have been tested for anti-inflammatory activity by our group in animal models. They successfully compete for iron with apotransferrin and under certain conditions exceed desferrioxamine in their iron scavenging abilities.[53]

Effect of iron on cells within the inflamed joint

LYMPHOCYTES

One of the earliest changes in the rheumatoid synovium is an infiltration of lymphocytes,[54] which are predominantly T cells.

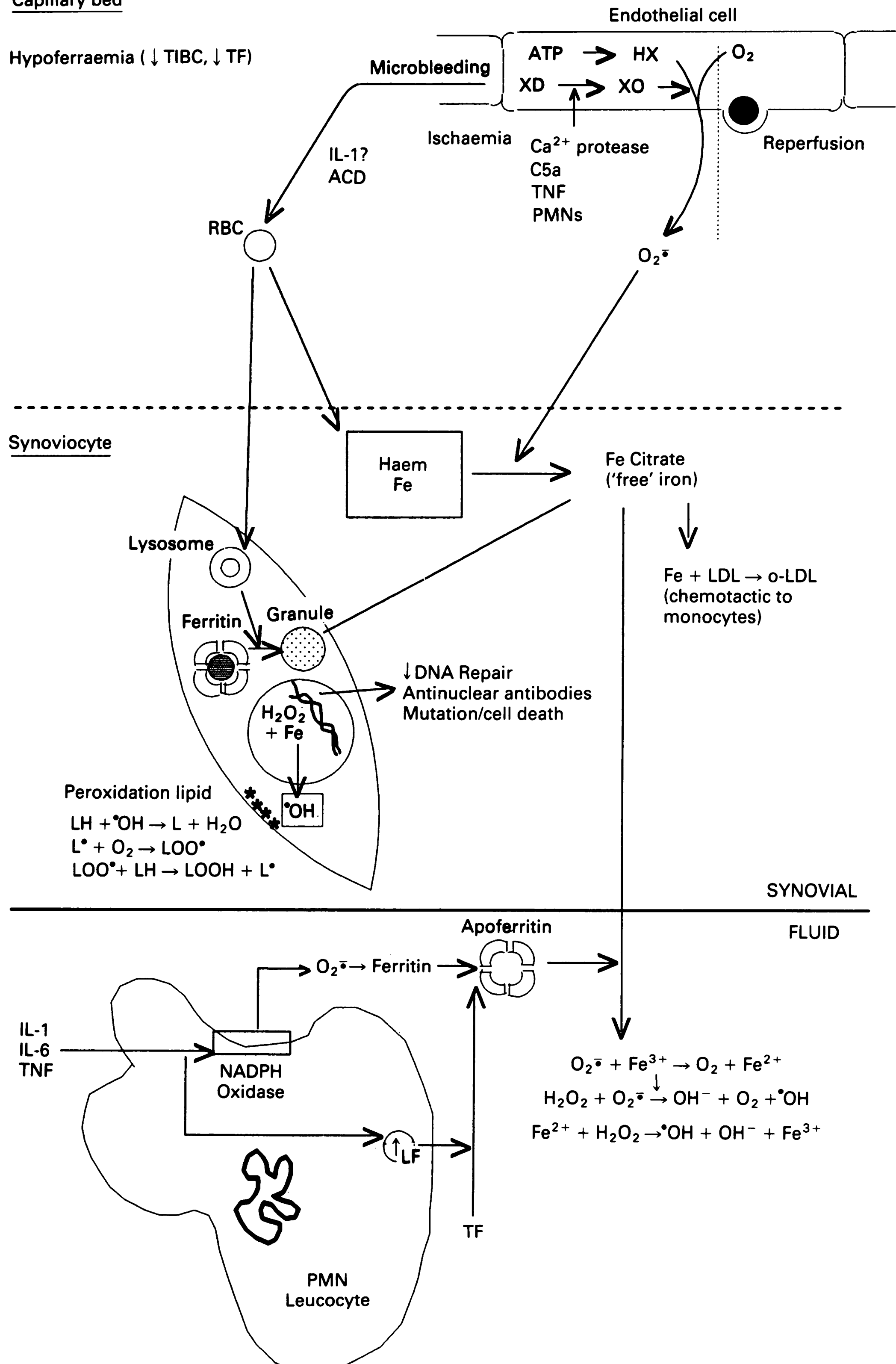
It is now generally agreed that lymphocytes require iron for proliferation and that this iron is obtained from transferrin.[55–57] Transferrin receptors are present in phytohaemagglutinin stimulated lymphocytes,[58] and proliferating lymphocytes acquire iron by a mechanism similar to that described for other cells. Iron uptake was shown to precede DNA synthesis,[59] probably because production of the ribonucleotide reductase, an enzyme containing iron, is a rate limiting step in lymphocyte proliferation.[60]

In vitro iron had a modulating effect on the expression of some human lymphoid cell surface markers.[61] In 1986 Bryan and coworkers studied the effect of ferric citrate on the expression of the surface markers on lymphocytes activated by pokeweed mitogen. They showed that iron suppresses the expression of the molecules identified by the monoclonal antibody OKT9, and had no effect on other activation associated markers such as Ia and T10. The authors concluded that iron has a differential immunoregulatory influence on the expression of certain lymphocyte surface molecules on actively dividing lymphocytes.[62] In addition, ferric citrate has been shown to diminish the cloning efficiency of human memory T4+ lymphocytes.[63] Iron also has a modulatory effect on immunological functions, as shown by Keown and Descamps. The authors demonstrated a marked inhibition of T cell immune responsiveness after the endocytosis of opsonised erythrocytes by adjacent macrophages in culture. This effect was not due to phagocytosis alone, similarly treated erythrocyte ghosts being without effect, but was traced to the inhibitory effect of an intracellular erythrocyte component.[64] Further work by this group showed that this substance is haemoglobin, and that ferritin and iron (III) salts also exert an immunosuppressive effect.[65] In an immunocytochemical study of the changes in rat synovial T cell subsets after a transient iron overload (provoked by a single intravenous injection of ferric citrate, which caused a transient increase in the transferrin saturation above 100%) De Sousa *et al* (1988) noted a relatively higher increase in the ingress of W3/25+ (CD4) than OX8+(CD8) cells in the synovium at 24 hours after the injection.[66] These findings illustrate and reinforce the apparent 'specific' response of the helper inducer T cell subset to changes in serum iron concentration. Roberts and Davies have postulated that the exacerbation of synovitis seen in rheumatoid patients given intravenous iron dextran may be related to an increase in lymphocyte proliferation in the synovial membrane.[67] Hence it seems that iron can either promote or suppress lymphocyte proliferation depending on its exact form and concentration in the rheumatoid synovium.

PHAGOCYTIC CELLS

The increased susceptibility to infection of patients with an iron overload as in the inherited disorder idiopathic haemochromatosis, or in patients receiving repeated transfusions, is attributed to the high plasma and tissue iron concentration, which creates an environment favourable for bacterial growth,[68] and to the impaired function of monocytes and neutrophils.[69] In patients receiving haemodialysis with iron overload related to multiple blood trans-

fusions Flament *et al* reported an impairment of phagocytosis together with a decrease in $O_2^{\bullet}$ production by neutrophils.[70] In addition, there is a decrease in the lytic ability of peripheral blood monocytes from patients with thalassaemia major, which negatively correlates with serum ferritin concentration.[71] Chemotaxis was decreased by $FeCl_3$ in vitro[72] and by post-treatment serum samples from patients given iron dextran.[73] Chemotaxis of neutrophils from patients with iron overload was also impaired.[69] Such impairment of neutrophil function is probably mediated by non-transferrin bound iron.[74] Hoepelman and coworkers performed a



Schematic representation showing probable sources of reactive oxygen species and iron within the joint and their consequences. TIBC=total iron binding capacity; TF=transferrin; TNF=tumour necrosis factor; PMNs=polymorphonuclear leucocytes; IL-1=interleukin 1; ACD=the anaemia of chronic disease; RBC=red blood cells; o-LDL=oxidised low density lipoprotein.

series of studies on the effects of iron on phagocyte function, aggregation, and adhesion to endothelial cells. They showed that when ferric citrate, in concentrations comparable with those found in the serum of patients with iron overload is incubated with neutrophils from healthy donors it impairs phagocytic function. The uptake of radiolabelled *S aureus* was used to assess the phagocytic function of neutrophils.[75] The authors postulated that iron can be released from ferric citrate by reduction to the water soluble ferrous state after interaction with $O_2^{\bar{\cdot}}$ or ascorbate in a mechanism similar to the release of iron from ferritin.[40] This led them to study the effect of ferrous iron on neutrophil phagocytic function. Ferrous ascorbate significantly impaired the phagocytic function of neutrophils at iron concentrations as low as 1–10 μmol/l. This toxic effect was inhibited by desferrioxamine and transferrin. Toxicity mediated by ferrous ammonium sulphate, but not by ferrous ascorbate, was prevented by the oxygen free radical scavengers thiourea, mannitol, and catalase. Ferrous iron impaired the uptake of *S aureus* by neutrophils of a patient with chronic granulomatous disease, whereas ferric iron did not. The authors therefore concluded that iron mediated impairment of neutrophil function is not only a result of the generation of reactive oxygen species but also of direct interaction of iron (II) or an iron (II) oxygen intermediate with molecules of the cell membrane.[76] All the iron complexes which were shown to impair phagocytic function also stimulated lipid peroxidation in both monocytes and neutrophils. The lowest concentration of ferrous ascorbate capable of inducing lipid peroxidation, however, was considerably higher than that which impaired phagocyte function.[77]

Further studies by Hoepelman and coworkers have shown that non-transferrin bound ferrous iron can induce neutrophil aggregation and adherence of neutrophils to endothelial cells.[78] This phenomenon can be of considerable importance, especially when there are high concentrations of non-transferrin bound iron in tissues—for example, in the synovial fluid of patients with RA.[37] Neutrophil-endothelium adhesion can lead to injury of the endothelium and may contribute to the exacerbation of the synovitis in rheumatoid patients after intravenous iron dextran infusion. In addition, the $O_2^{\bar{\cdot}}$ anion, released from stimulated neutrophils, may induce ferrous iron release from the endothelial cell itself and hence further augment neutrophil mediated damage to these cells. Iron induced neutrophil endothelial cell adherence could be completely inhibited by catalase or desferrioxamine. Hoepelman and coworkers have shown that adherence of neutrophils to endothelial cells, induced by iron, is primarily mediated by translocation of the specific granule protein lactoferrin to the plasma membrane. In addition, monoclonal antibodies directed against the leucocytes cell adhesion molecules (CD18) also inhibited iron mediated adherence. Iron also induced an increase in the surface expression of CD18 on neutrophils.[79]

In summary, non-transferrin bound iron is taken up by phagocytes and induces peroxidation of membrane lipids. This damage is mediated by the formation of the $^{\bullet}OH$ radical. In a highly reducing environment, however, as exists when ascorbic acid is present or when neutrophils are activated, damage is probably also mediated by other iron-oxygen intermediates. These free radicals induce peroxidation of membrane lipids, but other mechanisms such as changes in the cytoskeleton by damage to cell proteins may also impair phagocytic function. Iron mediated lipid peroxidation may also lead to translocation of lactoferrin and adhesion antigens leading to damage to endothelial cells and further release of iron and reactive oxygen species. Iron is present in the rheumatoid synovial membrane mainly in the form of ferritin. Ferritin is also found in the synovial fluid, where its concentration correlates closely with levels of other indices of intra-articular disease activity, such as synovial immune complexes. Iron concentrations within the synovial membrane do not correlate closely with the duration of the disease. This may be because such iron originates, at least in part, from microbleeding. Iron concentrations do correlate, however, with erosive bone damage. In early rheumatoid disease the presence of ferritin and haemosiderin iron in the synovial membrane implies a poor prognosis.[31] Oral iron supplements or total dose infusion of iron dextran exacerbate the rheumatoid synovitis. This exacerbation is thought to be due to the low molecular weight iron chelates which promote free radical production with subsequent lipid peroxidation, leading to cell damage and death.

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