Effect of nutritional iron deficiency on acute and chronic inflammation

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SUMMARY  Mild nutritional iron deficiency significantly reduced the severity of adjuvant induced joint inflammation assessed by histology, microfocal radiography, and subjective scoring. Other indices of adjuvant induced inflammation, such as increased levels of serum acute phase reactants, characteristic pathological changes in the inguinal lymph nodes, and granuloma formation at the site of adjuvant injection, were not different from those of control animals. The level of iron deficiency used did not affect the acute inflammatory response to a range of irritants and was not sufficient to suppress immune function. Both the hypersensitivity reaction to oxazalone and lymphocyte tritiated thymidine incorporation were normal. These observations support the view that iron has a selective influence on joint mediated inflammation.

The potential importance of iron in rheumatoid disease is well recorded.1 Any sustained inflammatory reaction causes changes in iron metabolism, with a drop in serum iron and a redistribution of iron to the activated reticuloendothelial system including, in rheumatoid disease, the inflamed synovium. Muirden and Senator suggested that this synovial iron deposition contributed to the joint inflammation,2 and Blake et al found that high levels of synovial iron anticipated a poor prognosis in early rheumatoid patients.3 Treatment of the anaemia associated with rheumatoid disease with intravenous iron dextran and with oral iron has been shown to exacerbate joint symptoms.4,5

Removal of deposited iron may, therefore, be expected to reduce inflammation and in support of this the iron chelator desferrioxamine has anti-inflammatory properties in a variety of models of inflammation.6,7 Recently we have shown that desferrioxamine treatment also significantly suppresses adjuvant disease in the rat.8 Adjuvant disease is a well recorded model, in which there is a local inflammatory response at the site of injection,9 generalised focal arthritis, synovitis and tendonitis10 accompanied by systemic lesions affecting the eyes, skin, urogenital and gastrointestinal systems.11 Characteristic pathological changes occur in the lymph nodes draining the injection site,12 and there is an increase in serum acute phase reactants13 and peripheral blood neutrophils.14 The inflamed synovium has also been shown to contain iron deposits.15 Desferrioxamine treatment appeared to reduce the joint symptoms of adjuvant disease specifically without affecting the systemic and local sequelae. Desferrioxamine, however, will chelate a range of other metals.16 In view of this we have extended our studies to look at the effect of mild nutritional iron deficiency on adjuvant disease and on models of acute inflammation not involving the joint. As iron deficiency may be associated with immune suppression we have also examined the effect of nutritional iron deficiency on lymphocyte proliferation and cell mediated immunity.

Materials and methods

Postweaned, male Wistar rats were obtained from Bantin and Kingman (Hull, UK). The test rats for each experiment were fed a commercial iron deficient diet (Volac, Royston, UK) with an iron concentration of 20 ppm. The control rats were maintained on the same diet plus iron supplementation in the form of ferrous sulphate to give a total iron concentration of 120 ppm. This corresponds with the recommended iron concentration found in commercial rat and mouse feed (Heygate & Sons, Northampton, UK). Both test and control rats in each experiment received food and triple distilled water freely.
All rats were weighed at weekly intervals. After five weeks measurement of haemoglobin levels using a cyanmethaemoglobin technique (Sigma, USA) confirmed that a mild iron deficiency had been established in the test rats. The rats were then divided into groups of 12 iron deficient (test) and 12 iron supplemented (control) rats in each experiment. At the end of each experiment haemoglobin levels were reassessed and samples of liver were removed for measurement of hepatic iron, copper, and zinc concentrations by atomic absorption.

**CHRONIC INFLAMMATION**

Adjuvant disease was induced on day 0 in iron deficient and control rats by the method of Kaibara et al.17 All rats received a single intradermal injection in the base of the tail of 0.1 ml of a 10 g/l solution of *Mycobacterium butyricum* (Difco, Surrey, UK) in liquid paraffin oil. Joint inflammation was assessed by an established method based on the foot scoring system first described by Currey and Ziff.18 A subjective score of 0–4 was allocated to each hind foot depending upon the extent of inflammation, with a maximum score of eight per rat. Scoring was as follows: 0 = no inflammation, 1 = redness and swelling of the foot, 2 = swelling of the foot such that the tendons were no longer visible, 3 = swelling extending to the ankle joint, and 4 = gross inflammation and deformities of the ankle joint. To eliminate bias scoring was carried out by two independent observers, one of whom had no knowledge of the treatments used in this experiment (see statistics).

All rats were killed on day 21, and the hind ankle joints were removed for histology and radiography. Joint tissue was decalcified and processed by a standard histological procedure. Paraffin sections (5 μm) were cut and stained with haematoxylin and eosin. Sections from each rat joint were assessed independently by the authors and by Dr G Kondratowicz (Department of Pathology, University of Birmingham). Joints were x rayed using microfocal radiography. The x ray system used in this work comprised a demountable x ray unit, an X–Y positioning frame, and a Marconi image intensifier and monitor. The special 25 μm microfocal x ray set had an effective resolution of >20 line pairs/mm. Radiographs of each rat ankle joint were obtained at a magnification of ×10 using standard x ray cassettes positioned in front of the image intensifier. Exposures were 40 kV (0.4 MA). Radiographs were assessed independently by the authors and by Dr N Evans (Selly Oak Hospital, Birmingham).

The primary inflammatory response at the base of the tail was assessed by measuring the diameter of the lesion. Serum acute phase reactants were measured using a COBAS B10 centrifugal microanalyser, and levels were compared with those of six normal rats of the same strain, sex, and age. Differential leucocyte counts were made of fresh blood smears removed from each rat. The inguinal lymph nodes were removed from each rat and processed by a standard histological procedure. The hyperplastic response of the lymph node to adjuvant was assessed semiquantitatively by measuring the maximum diameter of the nodes and determining the presence or absence of secondary follicles, paracortical hyperplasia, sinus histiocytosis, and dilatation and granuloma formation.

**ACUTE INFLAMMATION**

*Carrageenan pleurisy*

Carrageenan pleurisy was induced in iron deficient and control rats by the method of Velo et al.19 Rats were killed and exudate collected at five hours after induction of pleurisy.

*Urate pleurisy*

Pleurisy was induced using 0.2 ml of a 1% suspension of sodium biurate in phosphate buffered saline (PBS) following the above method.

*Pyrophosphate foot pad*

Foot pad oedema was induced using 0.2 ml of a 1% suspension of calcium pyrophosphate in PBS injected into the left hind foot pad. Foot pad oedema was assessed by measuring the circumference of the inflamed left foot at 1, 2, 3, 4, and 24 hours postinjection and subtracting the value for the non-inflamed right foot.

**IMMUNOLOGY**

*Oxazalone hypersensitivity*

Sensitisation and contact challenge with oxazalone was carried out by the method of Kishore et al.20 Ear oedema was measured at 0, 24, and 48 hours postchallenge using a micrometer.

*Lymphocyte proliferation*

Both iron deficient and control cells were cultured in both iron deficient and control serum. Cultures were carried out in sterile microplates in triplicate with 1.5x10⁶ cells in 100 μl RPMI containing 10% rat serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin or concanavalin A 9 μg/ml (Pharmacia, Sweden) or phytohaemagglutinin 280 μg/ml (Difco labs, USA).

The preparations were incubated for 66 hours at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. After 48 hours of
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Rats fed on the iron deficient diet had significantly reduced haemoglobin levels compared with the controls (115.7 (9.4) g/l vs 131.3 (8.4) g/l, p<0.01 (ST)). Iron deficient rats had significantly reduced hepatic iron stores compared with controls (35.7 (7) mg/kg vs 186 (41) mg/kg, p<0.001 (ST)). Hepatic copper and zinc levels were unaffected by the iron deficiency. (Copper levels--iron deficient 5.3 (1.7) mg/kg, control 5.2 (1.7) mg/kg; zinc levels--iron deficient 28.3 (8.9) mg/kg, control 25.9 (7.1) mg/kg (ST)).

**CHRONIC INFLAMMATION**

Initial signs of arthritis were noted from day 12. From day 12 to 14 there was no significant difference in joint score between the iron deficient and control rats. By day 15 the control animals had developed an intense destructive polyarthritis, while joint inflammation in the iron deficient group was significantly reduced (Fig. 1). There was no significant difference between the scores of the two observers as assessed by the Wilcoxon signed rank test.

All the control rats had evidence of periarticular...
connective tissue inflammation with an infiltrate consisting mainly of polymorphs with a few lymphocytes, histiocytes, and mast cells. All of the sections showed synovitis, bursitis, infiltration of the synovium with polymorphs, and exudation into the synovial space. Half of the control animals had evidence of fibroblastic proliferation and granulation tissue formation with areas of bone resorption, osteoblastic proliferation, and disorganised new bone formation. In contrast, two iron deficient rats showed no signs of joint disease, and the inflammation observed in eight of the rats was restricted to a mild synovitis and periarticular connective tissue inflammation. Interestingly, the infiltrate consisted of a mixture of polymorphs and lymphocytes, the numbers of polymorphs being greatly reduced compared with those observed in the control sections. Only two animals had developed bone erosions.

These results were reflected in the microfocal radiographs, where all of the control rats had evidence of soft tissue swelling. Half of these animals had osteoporosis of the tibial head, and four rats had florid new bone formation and periostitis. Only two iron deficient rats showed any signs of bone changes and these were similar to those of the control animals.

Both iron deficient and control rats developed a primary inflammatory focus at the site of injection in the base of the tail. There was no significant difference in the diameter of the lesion (iron deficient 7.00 (5.5) mm, control 4.67 (3.57) mm (ST)). Both iron deficient and control rats showed significant changes in serum acute phase reactants compared with rats of the same age, strain, and sex with no adjuvant disease. There was no significant difference between the two experimental groups except for total iron binding capacity (TIBC) (Table 1). Serum iron levels were reduced in control rats to the level in the iron deficient rats as a result of adjuvant disease.21 Both iron deficient and control rats had a significantly higher percentage of neutrophils in the peripheral blood compared with rats with no adjuvant disease (iron deficient 50 (8)% neutrophils, 46 (8)% lymphocytes, control 53 (9)% neutrophils, 42 (7)% lymphocytes; normal rats 19 (6)% neutrophils, 79 (5)% lymphocytes, p<0.001 (ST)).

Inguinal lymph nodes from both iron deficient and control rats showed a similar response to the adjuvant. There were no significant differences in the maximum diameter of the nodes (iron deficient 4.3 (1.0) mm, control 4.0 (0.5) mm (ST)), presence or absence of paracortical hyperplasia, sinus histiocytosis and dilatation, or the proportion of nodes in each group showing secondary follicle formation (iron deficient 75%, control 66% (MW)). The control rats, however, had significantly fewer granulomata in the lymph nodes (25% controls, 66% iron deficient, p<0.005 (MW)).

### Table 1 Change in serum acute phase reactant as a result of adjuvant disease in iron deficient and control rats compared with rats with no disease

<table>
<thead>
<tr>
<th>Test</th>
<th>Iron deficient</th>
<th>Control</th>
<th>No adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (μmol/l)</td>
<td>25.1 (3.6)</td>
<td>28.8 (6.6)</td>
<td>35.1 (5.7)*</td>
</tr>
<tr>
<td>TIBC (μmol/l)</td>
<td>108 (10)†</td>
<td>97 (6)</td>
<td>95 (6)**</td>
</tr>
<tr>
<td>Haptoglobin (g/l)</td>
<td>2.34 (0.6)</td>
<td>2.19 (0.8)</td>
<td>0.47 (0.17)*</td>
</tr>
<tr>
<td>Caeruloplasmin (mg/l)</td>
<td>1033 (194)</td>
<td>892 (266)</td>
<td>345 (22)*</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>26.1 (1.8)</td>
<td>25.9 (2.0)</td>
<td>31.9 (1.2)*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.5 (0.2)</td>
<td>1.7 (0.2)</td>
<td>1.3 (0.3)***</td>
</tr>
<tr>
<td>Seromucoid (g/l)</td>
<td>12.7 (5.2)</td>
<td>11.5 (6.5)</td>
<td>4.33 (0.7)*</td>
</tr>
</tbody>
</table>

Values are mean (SD).
Statistics—Student’s t test, n=12 in each group.
*Iron deficient compared with rats with no disease: *p<0.001, **p<0.1, ***p<0.05.
†Iron deficient TIBC (total iron binding capacity) compared with control p<0.01.

### Acute Inflammation

**Carrageenan pleurisy**

There was no significant difference in exudate volume (iron deficient 1.00 (0.14) ml, control 0.99 (0.09) ml (ST)) or the exudate total leucocyte count (iron deficient 8.58 (16)×10⁶ cells/ml, control 8.56 (21)×10⁶ cells/ml (ST)).

![Fig. 2 Development of foot pad oedema after injection with calcium pyrophosphate crystals. □ Iron deficient, ○ control.](image-url)
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Table 2  Resting and mitogen stimulated \(^3\)H incorporation measured in iron deficient (test) and control cells and sera

<table>
<thead>
<tr>
<th>Cells</th>
<th>Serum</th>
<th>Resting (cpm)</th>
<th>PHA 100 (\mu)l (cpm)</th>
<th>PHA 50 (\mu)l (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>Control</td>
<td>52 (7) 28583 (212)</td>
<td>16534 (3026)</td>
<td></td>
</tr>
<tr>
<td>Test</td>
<td>Test</td>
<td>50 (7) 27705 (1307)</td>
<td>13793 (2235)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>48 (6) 17522 (1381)</td>
<td>13742 (482)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td>40 (10) 17958 (1225)</td>
<td>12973 (368)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells</th>
<th>Serum</th>
<th>Resting (cpm)</th>
<th>Con A 50 (\mu)l (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>Control</td>
<td>60 (6) 17913 (6379)</td>
<td></td>
</tr>
<tr>
<td>Test</td>
<td>Test</td>
<td>58 (7) 19644 (2312)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>37 (5) 14050 (600)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td>45 (2) 14981 (731)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (SD).

Con A = concanavalin A; PHA = phytohaemagglutinin.

Urate pleurisy

There was no significant difference in the exudate volume (iron deficient 1-03 (0-07) ml, control 1-00 (0-14) ml (ST)) or the exudate total leucocyte count (iron deficient 80-86 (26)\(\times\)10\(^6\) cells/ml, control 82-20 (19)\(\times\)10\(^6\) cells/ml (ST)).

Pyrophosphate foot pad

Iron deficiency did not significantly affect the development of foot pad oedema induced by calcium pyrophosphate crystals (Fig. 2).

Immunology

Oxazalone hypersensitivity

There was no significant difference in ear oedema in response to challenge with oxazalone between iron deficient and control rats.

Lymphocyte proliferation

Iron deficiency did not significantly affect \(\[^3\]H\)thymidine incorporation by resting and mitogen-stimulated lymphocytes (Table 2).

Discussion

Nutritional iron deficiency has traditionally been described as detrimental to host defences against disease. In recent controversies a possible protective role against heart disease, cancer, and both bacterial and parasitic infections has been attributed to it.

Our results suggest a selective beneficial effect of nutritional iron deficiency in reducing joint inflammation in the adjuvant model. Iron deficiency did not affect the development of the primary inflammatory focus at the site of injection, however, nor the systemic complications characteristic of adjuvant disease, such as raised acute phase reactants, altered peripheral blood lymphocyte, neutrophil ratios, and inguinal lymph node hyperplasia. The same levels of iron deficiency had no influence on the acute inflammatory response to carrageenan, sodium biurate, or calcium pyrophosphate.

Nutritional iron deficiency has been associated with a suppression of immune function. Iron deficient patients are reported to have a decreased percentage of T lymphocytes, impaired incorporation of \(\[^3\]H\)thymidine by stimulated lymphocytes in culture, and a depressed delayed hypersensitivity response. Nutritionally iron deficient rats have been reported to have impaired humoral and cell mediated immunity. The lymphocyte abnormalities may result from defective DNA synthesis and cell proliferation due to a reduced activity of the iron containing enzyme ribonucleotide reductase.

The modest level of iron deficiency produced in our experiments, however, did not affect the development of oxazalone hypersensitivity or lymphocyte \(\[^3\]H\)thymidine incorporation.

Nutritional iron deficiency has been associated with abnormalities in neutrophil function, though phagocytic function may remain normal. Iron deficient neutrophils have been shown to be defective in the oxidative reduction of nitroblue tetrazolium dye by the superoxide radical, suggesting the activity of the iron containing enzyme NADPH oxidase is reduced. In addition, the activity of myeloperoxidase, an important bactericidal system which contains iron, may also be adversely affected by iron deficiency.

Iron is an important catalyst in the production of the most toxic oxygen radical \(\mathrm{OH}^+\) from superoxide and hydrogen peroxide by the Fenton reaction.

\[
2\mathrm{O}_2^- + 2\mathrm{H}^+ \rightarrow \mathrm{H}_2\mathrm{O}_2 + \mathrm{O}_2
\]

\[
\text{Fe}^{3+} + \mathrm{H}_2\mathrm{O}_2 \rightarrow \text{Fe}^{3+} + \mathrm{OH}^+ + \mathrm{OH}^-
\]

During inflammation it is thought that oxygen radicals produced by stimulated phagocytic cells contribute to tissue damage by inducing lipid peroxidation. There is evidence that such a process may contribute to joint inflammation as lipid peroxidation products have been found in synovial fluid both from rheumatoid patients and rats with adjuvant arthritis. In both diseases iron deposits occur in the inflamed synovium and in rheumatoid synovial fluid iron is present in a form capable of catalysing the Fenton reaction. Inhibitors of the superoxide radical are reported to significantly reduce joint inflammation and the production of lipid peroxidation products in adjuvant disease. The presence of stimulated phagocytic cells in the
inflamed area provides one mechanism for the generation of oxygen radical damage. It is of interest that there were fewer neutrophils in the joint tissue of iron deficient rats in our experiments than in the controls, though the percentage of neutrophils in the circulation remained high. This is unlikely to be related to a straightforward reduction in neutrophil recruitment in iron deficiency as there was no significant reduction in neutrophil counts in the pleural exudate from iron deficient rats.

Although oxygen radical production during inflammation is by no means specific to the joint, there is evidence that the joint provides a unique environment that may account for the specificity of our results. We have previously shown that exercise of the inflamed rheumatoid knee joint results in significant intra-articular pressure increases and decreased synovial fluid oxygen tension. When the joint was rested the intra-articular pressure returned to normal resting levels but the synovial fluid oxygen tension increased. Synovial fluid fluorescent IgG levels (thought to be formed in vivo by free radical mediated damage to the IgG molecule) varied with those of the oxygen tension.

McCord was the first to show that ischaemia followed by reperfusion of the tissues resulted in metabolic changes during the ischaemic period leading to the production of oxygen free radicals during reperfusion. We have postulated that such a process occurs in the inflamed joint. Ischaemia results in the delocalisation of cellular protein bound iron to smaller chemical species capable of catalysing free radical mediated lipid peroxidation. Iron chelation using desferrioxamine has been shown to significantly reduce the tissue damage following cardiac arrest. It is possible that nutritional iron deficiency specifically reduces joint inflammation by reducing the available iron required for oxygen free radical production. Further support for the ischaemia/reperfusion hypothesis comes from the observation that bed rest or splinting of inflamed joints is clinically beneficial and that immobilisation of the limbs by sciatric denervation significantly reduced the severity of joint symptoms in adjuvant disease.

We have shown that nutritional iron deficiency has a beneficial effect in reducing joint inflammation in the adjuvant model without affecting the systemic components of the disease or the models of acute inflammation. This observation suggests an apparently selective influence of iron on joint mediated inflammation.

References

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